KU Leuven
Biomedical Sciences Group
Faculty of Medicine
Department of Oncology

EXPLORATION AND DEVELOPMENT OF COMBINATION IMMUNOTHERAPY AS A TREATMENT APPROACH FOR UTERINE CANCER

Anke VANDERSTRAETEN

Jury:

Promoter: Prof Dr. F. Amant
Co-promoter: Prof. Dr. S. W. Van Gool
Prof. Dr. K. Thielemans
Chair:Prof. Dr. J. Swinnen
Secretary: Prof. Dr. H. Wildiers

Jury members: Prof. Dr. D. Bullens
Prof. Dr. K. Vermaelen
Prof. Dr. J. de Vries

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Biomedical Sciences

October 30th 2014

Dankwoord

Deze thesis wordt voorgedragen aan de KU Leuven met als rector Prof Dr. Rik Torfs en als vice-rector van de groep biomedische wetenschappen Prof. Dr. Wim Robberecht, aan de faculteit geneeskunde met als decaan Prof. Dr. Jan Goffin.

In de eerste plaats richt ik een woord van dank aan mijn promotor, Prof. Dr. Frederic Amant. Mijn interesse voor immunologie is reeds vrij vroeg in mijn opleiding begonnen en groeide gaandeweg. Tijdens de stage voor mijn masterthesis kwam ik voor het eerst gedurende een langere periode in contact met hands-on immunologisch onderzoek en kreeg ik ook de microbe voor dit soort onderzoek te pakken. Hiermee zou ik hem willen danken dat ik de kans kreeg om mijn doctoraat in het immunologisch onderzoek verder te zetten. Om dezelfde reden zou ik ook het Antikankerfonds en met name Lydie Meheus en Luc Verelst oprecht willen danken. Ook richt ik graag een woord van dank aan mijn co-promotoren, Prof. Dr. Stefaan Van Gool en Prof. Dr. Kris Thielemans voor hun ondersteuning tijdens mijn doctoraat.

In het bijzonder zou ik twee van mijn collega's specifiek willen danken. Eerst en vooral Sandra. Het dikke pak werk wat achter dit dankwoord gebundeld is ook gebundeld denk- en onderzoekswerk, waar ook jij een grote bijdrage aan leverde. Bedankt voor de wetenschappelijke ondersteuning voor dit werk en niet te vergeten de vele (late) uurtjes nalees- en verbeterwerk.
De volgende in mijn 'dankjewel'-rijtje is Tina, stilaan ook wel gekend als 'collegaatje'. Bedankt voor de fijne samenwerking en de plezante momenten op het labo.
Ik zou jullie beiden ook heel erg willen bedanken voor de bemoedigende niet werk-gerelateerde babbels en ventileermomentjes die er in verschillende richtingen geweest zijn.
We zijn alle drie door Diego in zijn dankwoord vorig jaar omgedoopt tot de 'immuno girls' en ik denk dat we mogen stellen dat de samenwerking tot hier toe toch vrij goed loopt. Ook na mijn doctoraat zullen die 'immuno girls' of het 'team immuno' nog eventjes in deze samenstelling blijven bestaan. Het klinkt een beetje als een of andere vreemde voetbalploeg, maar ik heb met mijn rode lantaarn positie tijdens de "WK 2014" pronostiek denk ik bewezen dat ik over niet al te veel voetbalkennis beschik, dus dat moeten we misschien toch maar niet ambiëren… Ik ben er in elk geval blij om dat ik er ook nog eventjes deel van zal uitmaken.

Uiteraard zijn deze twee dames niet de enige collega's die een bedankje verdienen. Het zijn er te veel om jullie allemaal op te noemen, maar ik zou jullie allemaal enorm willen bedanken voor de leuke sfeer op het labo en voor alle steun die ik van jullie kreeg de laat ons zeggen minder leuke momenten…
Toch enkele specifieke extra bedankjes voor een aantal mensen. Lieve, Katrien, Rieta, bedankt voor de hulp bij het experimentele werk en alle handige tip en trucjes. Annick, bedankt om soms in allerijl nog stalen te verzamelen uit de tumorbank om analyses op te kunnen doen. Sileny, dankjewel voor alle leuke babbels en de toffe momenten, ook buiten het werk: een ijsje op de bank aan de vijver van Gasthuisberg, Italiaanse etentjes, sushi's, last-minute terrasjes, en natuurlijk niet te vergeten de pauze voor chocolade. Uiteraard mag een beetje het "manusje van al" van het labo niet ontbreken. Petra, dikke merci voor alle hulp in de administratieve mallemolen en alle overige bijstand wanneer nodig.

Ook absoluut niet te vergeten in dit dankwoord zijn mijn vrienden en familie. Zoals vele mensen hecht ik enorm veel belang aan beide. Ik zou hen elk apart welgemeend en oprecht willen

bedanken voor de steun die ik buiten het werk om van hen krijg en voor de interesse die ook vaak voor mijn onderzoek werd getoond.

Bij deze ben ik (hoop ik) rond en houd ik het (eindelijk ☺) voor bekeken wat het schrijven aan mijn thesis betreft.

Veel leesplezier!

Anke.

Table of contents

Abbreviations

µF	µ-Faraday
ACT	adoptive cell transfer
ACD	anticoagulant citrate dextrose
allo-MLR	allogeneic mixed leukocyte reaction
APAAP	alkaline phosphatase-anti alkaline phosphatase
APC	antigen presenting cell
APC (fluorochrome)	allophycocyanin
AWED	alive with evidence of disease
BORIS	brother of the regulator of imprinted sites
CA125	cancer antigen 125
CAR	chimeric antigen receptor
CD	cluster of differentiation
cDNA	copy DNA
CMV	cytomegalovirus
CR	complete remission
CRP	C-reactive protein
CS&T beads	cytometer setup and tracking beads
CTCAE	common terminology criteria for adverse events
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
CY (fluorochrome)	cyanin
CY	cyclophosphamide
DAB	3,3-diaminobenzidine
DAMP	damage-associated molecular pattern molecule
DC	dendritic cell
DC-LAMP	dendritic cell - lysosomal-associated membrane protein
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EBV	epstein-barr virus
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EMA	European medicines agency
EMCAR	endometrial carcinoma
FDA	food and drug administration
FIGO	international federation of gynecology and obstretics (fédération internationale de gynécologie et d'obstétrique)
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FSC	forward scatter
GM-CSF	granulocyte monocyte colony-stimulating factor
HE	hematoxylin-eosin
HIER	heat-induced epitope retrieval
HLA	human leukocyte antigen

HRP	horse radish peroxidase
hsCRP	high sensitive C-reactive protein
hTERT	human telomerase reverse transcriptase
iDC	immature dendritic cells
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IgG	immunoglobulin G
IHC	immunohistochemistry
IL	interleukin
IMDM	Iscove's modified Eagle's medium
irRC	immune-related response criteria
IU	international units
MAGE-A3	melanoma-associated antigen 3
mDC (DCm)	mature dendritic cells
MDSC	myeloid-derived suppressor cells
meta EMCAR	metastasis endometrial carcinoma
meta US	metastasis uterine sarcoma
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MMP	matrix metallo proteinase
MMR	mismatch repair
MNE	mean normalized expression
moDC	monocyte-derived dendritic cells
MR	mixed response
mRNA	messenger RNA
MUC1	mucin-1
NBT	nitro blue tetrazolium chloride
nl EM	normal endometrium
nm	nanometer
OD	optical density
p.o.	per os
PBMC	peripheral blood mononuclear cells
PD	progressive disease
PD-1	programmed death 1
PD-L1	Programmed death ligand 1
PD-L2	Programmed death ligand 2
PE	phyco-erythrin
PerCP	peridinin chlorophyll
PGE2	prostaglanding E2
PMA	phorbol 12-myristate 13-acetate
PR	partial remission
pr EMCAR	primary endometrial carcinoma
pr US	primary uterine sarcoma
qRT-PCR	quantitative real-time PCR
rec EMCAR	recurrent endometrial carcinoma

rec US	recurrent uterine sarcoma
RECIST	response evaluation criteria in solid tumors
rh	recombinant human
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
Sp17	sperm protein 17
SSC	side scatter
TAA	tumor-associated antigen
TAM	tumor-associated macrophage
TCR	T cell receptor
Teff	effector T cells
TGF	tumor growth factor
Th2	T helper 2
TIL	tumor-infiltrating lymphocytes
TLR	toll-like receptor
TMA	tissue microarray
TNF	tumor necrosis factor
Treg	regulatory T cells
US	uterine sarcoma
VNTR	variable nucleotide tandem repeat
WHO	world health organization
WT1	Wilms' tumor 1

Project situation

The most common type of gynecological malignancies is endometrial cancers [1]. Worldwide approximately 142 000 women are diagnosed with endometrial cancer each year, making this affliction the seventh most common malignant disorder. in 2008, 26.6/100 000 women were diagnosed, the highest incidence occurring in the Flemish region (29.1/100 000) [2]

There are two distinct types of endometrial cancer, designated type I and type II. Type I cancers, or endometrioid cancers, comprise approximately 80% of all endometrial cancers. This type bears a more benign phenotype and is usually associated with a favorable prognosis. In contrast, type II endometrial cancer has a stronger malignant phenotype and poor prognosis. The current first-line treatment for endometrial cancer is hysterectomy with or without lymphadenectomy [3, 4], often accompanied by (neo)adjuvant chemotherapy or radiotherapy [1, 4, 5]. An uncommon type of gynecological malignancies are the uterine sarcomas, representing only 3% of all uterine tumors [6]. Similar to endometrial cancer, surgery is the cornerstone of their treatment. However, with this type of tumor, little to no clinical benefit has been proven for adjuvant chemotherapy, radiotherapy or hormonal therapy.

Despite a fair cure rate for uterine tumors when diagnosed in an early stage, late stage disease has a much worse prognosis and is more prone to metastasis and relapse, which in the majority of cases leads to fatality even with robust treatment regimens. The 5-year survival rate for type II endometrial cancers is a mere 30% with a recurrence rate of 50% to 80% due to widespread metastatic disease [7, 8]. The mean overall survival for recurrent endometrial cancer is approximately 12 months. Consequently, there is an urgent need for better treatment modalities in uterine cancer, with as little as possible toxic side-effects or physical discomfort. The latter is especially important since in the majority of cases postmenopausal and older women are treated.

Immunotherapy, designated as breakthrough of the year in 2013 [9], is one possible treatment approach. During tumor growth, the immune system interacts with and attempts to eradicate the tumor. Immune therapy is based on the recognition of so-called tumor-associated antigens (TAA) by the immune system. TAA enable the immune system to discriminate malignant cells from their benign counterparts and raise a specific anti-tumor response without harming healthy surrounding tissue.[10].

One of the many possible immunotherapeutic treatment modalities is that of dendritic cell (DC) therapy. Altogether, DC vaccination has been reported to be nontoxic and encouraging clinical and immunological responses have been observed. However, further research is needed to obtain optimally activated DC capable of mediating tumor regression that correlates with the induction of an anti-tumor immune response. Moreover, the absence of toxicity argues for the inclusion of less advanced cancer patients and patients who have had prior tumor debulking surgery, in order to limit the effect of tumor-induced immunosuppression on DC, in DC vaccination trials.

The current project finds its main focus in the development of an immunotherapeutic treatment for uterine cancer, in which both the development of a DC vaccine and the validation of suitable target antigens to include in the vaccine are included. In addition, possibilities to modulate immunosuppressive mechanisms involved in uterine cancer are explored.

REFERENCES

1. Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E, Vergote I. Endometrial cancer. Lancet. 2005;366(9484):491-505.
2. http://www.kankerregister.org/.
3. Amant F, Neven P, Vergote I. Lymphadenectomy in endometrial cancer. Lancet. 2009;373(9670):1169-70; author reply 70-1.
4. Amant F, Mirza MR, Creutzberg CL. Cancer of the corpus uteri. Int J Gynaecol Obstet. 2012;119 Suppl 2:S110-7.
5. Coosemans A, Nik SA, Caluwaerts S, Lambin S, Verbist G, Van Bree R, et al. Upregulation of Wilms' tumour gene 1 (WT1) in uterine sarcomas. Eur J Cancer. 2007;43(10):1630-7.
6. Amant F, Coosemans A, Debiec-Rychter M, Timmerman D, Vergote I. Clinical management of uterine sarcomas. Lancet Oncol. 2009;10(12):1188-98.
7. Brooks N, Pouniotis DS. Immunomodulation in endometrial cancer. Int J Gynecol Cancer. 2009;19(4):734-40.
8. Ellenson LH, Wu TC. Focus on endometrial and cervical cancer. Cancer Cell. 2004;5(6):533-8.
9. Couzin-Frankel J. Breakthrough of the year 2013. Cancer immunotherapy. Science. 2013;342(6165):1432-3.
10. Aarntzen EH, Figdor CG, Adema GJ, Punt CJ, de Vries IJ. Dendritic cell vaccination and immune monitoring. Cancer Immunol Immunother. 2008;57(10):1559-68.

Part I General introduction

Part I

Chapter 1 General introduction

General introduction

Although described in the late 1950's, research focusing on the role of the immune system in cancer has long been sidetracked. However, in the last decades, the topic has come back into focus. The complexity of the immune system is reflected in its role in cancer. The immune system plays both a tumor promoting and tumor suppressing role, depending on the intratumoral composition of the immune system as well as immune evasion mechanism exploited by tumor cells.

The complex role the immune system plays in cancer is explained by the so-called immuno-editing theory, by Schreiber et al., describing tumor formation in three separate stages, all of which involve the immune system [1]. Although as research progresses, the complexity of the interplay still increases due to newly discovered players and their exact role in carcinogenesis, the theory describes three basic steps in the process of carcinogenesis. These steps may, but to do not necessarily involve, tumor formation: *elimination, equilibrium* and *escape* (Fig. 1). During the elimination phase, both the innate and the adaptive arm of the immune system cooperate in the process of tumor eradication. The exact signals that attract the immune system to the tumor are not fully elucidated but likely involve the release of so-called "danger signals" and stress ligands which are also involved in the initiation of an immune response to clear a pathogenic host or to initialize tissue repair. In case of tumor elimination, cancer cells are destroyed before they become clinically relevant. However, cancer cells cannot be eradicated in all cases. Cells that are not eradicated by the immune system can be kept under control in the *equilibrium* state. In this stage of carcinogenesis, tumor cells are kept in a state of dormancy, preventing the outgrowth of these tumor cells into established tumors. However, the way in which the immune system functions to control latent tumor cells, or a change in the controlling capacity of the immune system may in fact change the characteristics of the tumor cells, which may enable them to escape from immune control. In this stage of tumor immune *escape*, the immune system is no longer capable of tumor control, causing clinically apparent established tumors [1].
As exemplified in Fig. 1, many immune-related factors play an important role in tumor formation. Consequently, therapeutic intervention increasing the immune system's capacity to attack the tumor or circumventing the immunosuppressive mediators can be of importance in cancer treatment.
Many different forms of anticancer immunotherapy have already been investigated to date, comprising both active and passive forms of immunotherapy.
Passive immunotherapy consists of the administration of active immune components for direct antitumor effects.

Passive immunotherapy collectively includes:

- Immunization using antibodies
- Adoptive transfer of *in vitro* activated cells, e.g. T cells or NK cells
- Inhibition of immunosuppression
- (low-dose) chemotherapy

Active immunotherapy is aimed at stimulating the patients' immune response. Active immunotherapy regimens comprise:

- DNA vaccines
- Peptide vaccines
- Immunostimulatory cytokines
- Oncolytic viruses
- Dendritic cell-based vaccines

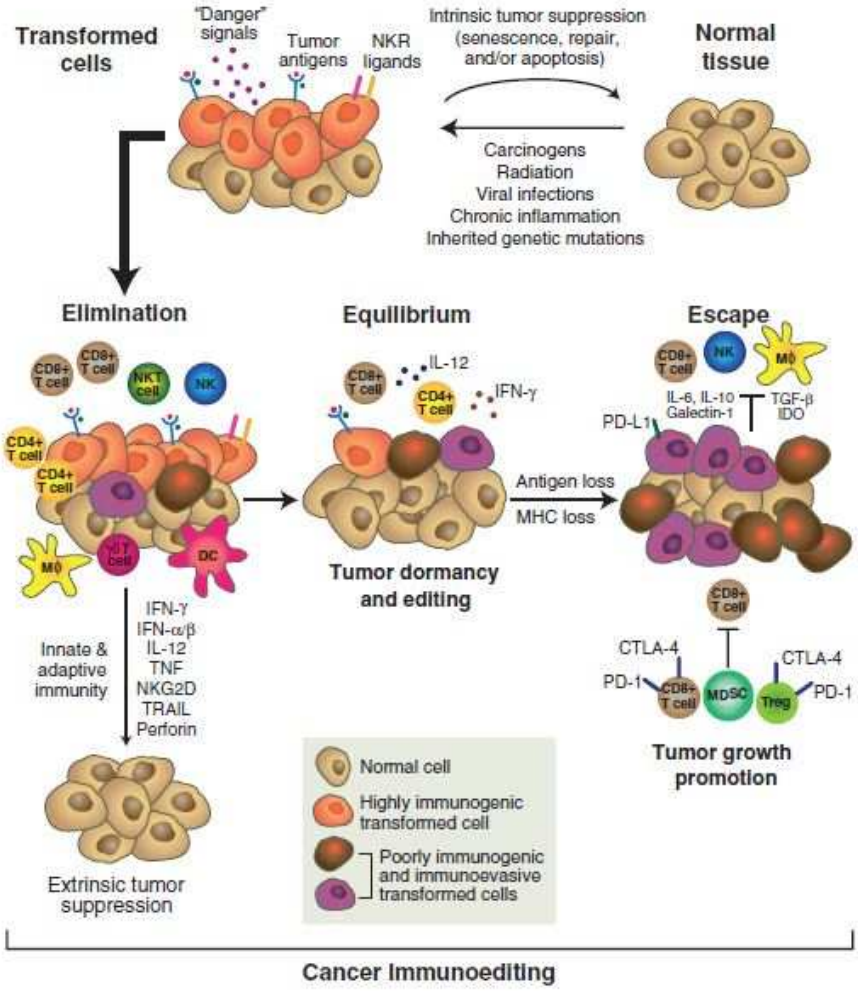


Figure 1: The cancer immuno-editing theory (adopted from Schreiber et al. [1]).

These different modalities and their disadvantages will briefly be discussed below, with a more elaborate analysis of different modalities of dendritic cell vaccination.

Passive immunotherapy

MONOCLONAL ANTIBODIES

Many different types of monoclonal antibodies have been developed, such as humanized antibodies, human antibodies using phage display and chimeric antibodies [2]. Nowadays, 15 different FDA-approved monoclonal antibodies are used in the clinic for the treatment of several cancer types (Table 1).

Table 1: FDA-approved monoclonal antibodies used in cancer therapy. (adapted from Vachelli et al. [3]).

mAb	Target	Approved	Type	Indications
Alemtuzumab	CD52	2001	Hzed IgG1	Chronic lymphocytic leukemia
Bevacizumab	VEGF	2004	Hzed IgG1	Glioblastoma multiforme, colorectal, renal and lung cancer
Brentuximab Vedotin	CD30	2011	C IgG1	Hodgkin's and anaplastic large cell lymphoma (coupled to MMAE)
Catumaxomab	CD3 EPCAM	2009	M-R hybrid	Malignant ascites in patients with $EPCAM^{+}$ cancer
Cetuximab	EGFR	2004	C IgG1	HNC and colorectal carcinoma
Denosumab	RANKL	2011	H IgG2	Breast cancer, prostate carcinoma, and giant cell tumors of the bone
Gemtuzumab Ozogamicin	CD33	2000	Hzed IgG4	Acute myeloid leukemia (coupled with calicheamicin)
Ibritumomab tiuxetan	CD20	2002	M IgG1	Non-Hodgkin lymphoma (coupled with ^{90}Y or ^{131}I)
Panitumumab	EGFR	2006	H IgG2	Colorectal carcinoma
Pertuzumab	HER2	2012	Hzed IgG1	Breast carcinoma
Ofatumumab	CD20	2009	H IgG1	Chronic lymphocytic leukemia
Rituximab	CD20	1997	C IgG1	Chronic lymphocytic leukemia and non-Hodgkin lymphoma
Tositumomab	CD20	2003	H IgG1	Non-Hodgkin lymphoma (naked or coupled with ^{131}I)
Trastuzumab (emtansine)	HER2	1998	Hzed IgG1	Breast carcinoma (naked or coupled to mertansine) and gastric or gastroesophageal junction cancer
Ipilimumab	CTLA-4	2011	H IgG1	Melanoma

HNC: head and neck carcinoma, Hzed: humanized, M: murine, C: chimeric, H: human, Ig: immunoglobulin.

Antibodies used in cancer therapy have several antitumor functions [2]. They can be used for inhibition of pro-survival cascades, interference with tumor-stroma interactions, which inhibits tumor cell growth, direct binding of tumor-associated antigens (TAA) and consequently, activation of immune mechanisms, and for targeting the crosstalk between the immune system and the tumor, for example by facilitating antigen presentation by DC.

ADOPTIVE CELL TRANSFER

Adoptive cell transfer (ACT) consists of the transfer of *ex vivo* activated effector cells in order to achieve direct antitumor effects. Cells most commonly used for transfer consist of antitumor T cells and NK cells. However, γδ T cells as well as NKT cells, mast cells, eosinophils and macrophages are valid candidates [4]. The source from which these cells can be retrieved or generated are either tumor tissue or peripheral blood. The source of the cells may be related to the objective response rate of the treatment, as exemplified in melanoma [5], where the use of the entire TIL population resulted in the highest objective response rate, whereas the use of selected individual clones resulted in the lowest response rate. Although generating substantial response rates, the use of tumor-infiltrating lymphocytes (TIL) for ACT requires the availability of tumor material and involves a cumbersome isolation procedure which is often not equally successful at generating tumor-reactive TIL [5]. In addition, the functional capacities of TIL are highly dependent on the culturing period and thus the "age" of the TIL used for transfer. The use of so-called "young" TIL, which have only had a brief culturing period has been shown to be result in comparable clinical responses as conventionally cultured TIL [6]. Alternative to the use of TIL, peripheral blood T cells can be used for the cloning of high avidity T cell receptors (TCR). These T cell receptors can be generated in large quantities and transfected into lymphocytes, thereby generating large quantities of antigen-specific T cells [7].

An important limitation of the use of TCR genes is the major histocompatibility complex (MHC) restriction [7]. T cells transfected with certain TCR genes can only be used in patients with a certain human leukocyte antigen (HLA) type. This limitation can be avoided by the use of chimeric antigens receptors (CAR). CAR consist of the antibody binding portion of an antibody, which does not comply to MHC restriction and the intracellular signaling domain of the TCR, which activates T cells. An additional advantage of the use of CAR is that it does not only recognize antigen peptides, as does the conventional TCR, but also other surface molecules, such as carbohydrates and glycolipids, which may be associated with tumors [7]. CAR technology has been used successfully in clinical trials in several cancer types, such as neuroblastoma, lymphoma, melanoma and colorectal cancer, summarized in [7].

ANTIBODY BLOCKADE OF IMMUNE CHECKPOINTS

In clinical trials using immunotherapy, there is an evident lack of a consistent correlation between the induction of an immune response and a clinical response. One of the reasons which are believed to be at the base of this discrepancy is the presence of several immune suppressive mechanisms in tumors. Consequently, strategies to suppress or circumvent these immunosuppressive mediators would preferentially be incorporated in clinical trials. Nowadays, such inhibitors are being evaluated (pre)clinically [8] (Table 2).

Table 2: Immune modulating antibodies currently in clinical development (adapted from Blank et al. [8]).

Target	Biological function	Antibody or fusion protein	State of clinical development
CTLA-4	Inhibitory receptor	Ipilimumab, fully human IgG4	EMA and FDA approved for melanoma, phase II and III trial in RCC, SCLC/NSCLC, prostate, pancreatic, ovarian, and Merkel cell carcinoma
		Tremelimumab, fully human IgG2	Failed in phase III for melanoma, phase II in mesothelioma
PD-1	Inhibitory receptor	Nivolumab, fully human IgG4	Phase III for melanoma and NSCLC recruiting, phase II in RCC
		Lambrolizumab (MK-3475), humanized IgG4	Phase III for melanoma and NSCLC recruiting, phase II in CRC
		Pidilizumab, CT-011, humanized IgG	Phase II in follicular lymphoma, DLBCL, multiple myeloma, AML, RCC, CRC, pancreatic cancer
		AMP-224 PD-L1 and human IgG1 fusion protein	No active study
PD-L1	Inhibitory ligand	BMS-936559 (MDX-1105), human IgG4	No recruiting study
		MEDI4736, engineered human IgG1	Phase I recruiting
		MPDL3280A, engineered IgG1	Phase II in melanoma, NSCLC
		MSB0010718C (Merck---Serono)	Phase I in solid tumors, recruiting
ICOS	Co-stimulatory receptor	MEDI570, fully human IgG1	Phase I terminated
		AMG557, fully human IgG2	Phase Ib in SLE and psoriasis
B7-H3	Inhibitory ligand	MGA271, humanized igG1	Phase Ib in melanoma
B7-H4	Inhibitory ligand		Preclinical development
LAG3	Inhibitory receptor	IMP321, recombinant soluble LAG3-Ig fusion protein	Phase II in melanoma
TIM3	Inhibitory receptor		Preclinical development
TNF(R) family			
CD40	Co-stimulatory receptor	CP-870,893, fully human IgG2	Phase Ib in melanoma (+ tremelimumab) and pancreatic cancer (+ gemcitabine)
		Lucatumumab, fully human IgG1	Phase II in lymphoma
		Dacetuzumab, (SGN040), humanized IgG1	
CD137	Co-stimulatory receptor	Urelumab (BMS-663513), fully human IgG4	Phase II in melanoma, phase Ib in NSCLC and B-NHL
		PF-05082566, fully human IgG2	Phase Ib in NHL (+ rituximab
CD27	Co-stimulatory receptor	CDX-1127, fully human IgG1	Phase I
OX40	Co-stimulatory receptor	Anti-CD40 mouse IgG	Phase I
OX40L	Co-stimulatory ligand	RO4989991, fully human IgG1	Phase II in allergic asthma
GITR	Co-stimulation	TRX518, engineered human IgG1	Phase Ib in melanoma

EMA: European medicines agency, FDA: food and drug administration, RCC: renal cell carcinoma, SCLC: small cell lung carcinoma, NSCLC: non-small cell lung carcinoma, CRC: colorectal cancer, DLBCL: diffuse large B cell lymphoma, AML: acute myeloid leukemia, SLE: systemic lupus erythematosus, B-NHL: B cell non-Hodgkin lymphoma, NHL: non-Hodgkin lymphoma.

(LOW DOSE) CHEMOTHERAPY

Chemotherapy is known to have immunosuppressive effects resulting from direct toxic effects on immune cells and bone marrow suppression. Nonetheless, chemotherapy can also have immunostimulating or immunomodulating effects, providing opportunities for immunotherapy. Chemotherapy has for example been shown to have an indirect activation effect on DC, and can result in depletion or functional suppression of regulatory T cells (Treg). The latter has been shown when using very low doses of cytotoxic chemotherapy. These low doses may also have a beneficial stimulatory effect on effector cells [9]. The immune stimulatory effects that are known for the different chemotherapeutic agents that are currently used in the clinic are summarized in Table 3.

Table 3: Immune stimulating effects of chemotherapy (adopted from Shurin et al. [9]).

Attenuation of immune tolerance		
	Elimination of regulatory T cells	cyclophosphamide
	Functional blockage of regulatory T cells	cyclophosphamide
	Depletion of myeloid-derived suppressor cells	gemcitabine
	Stimulation of differentiation of myeloid-derived suppressor cells into dendritic cells	paclitaxel
	Polarization of myeloid-derived suppressor cells toward M1 macrophages	docetaxel
	Re-polarization of regulatory dendritic cells into myeloid dendritic cells	paclitaxel
Direct stimulation of immune cells		
	Increase in the tumoricidal potential of macrophage and NK cells	doxorubicin cyclophosphamide
	Stimulation of maturation and function of dendritic cells	paclitaxel, methotrexate, doxorubicin
	Potential effect on IL-2 production by T helper cells	doxorubicin
Modulation of tumor cells		
	Restoration of tumor cell response to Fas-L and TRAIL and thus increase in NK cells and CTL-induced cytotoxicity	cisplatin, doxorubicin, mitomycin C, fluorouracil, and camptothecin
	Apoptosis of tumor cells, resulting in increased immunogenicity of tumor cells	doxorubicin, 5-fluorouracil, gemcitabine, paclitaxel
	Induction of expression of cell surface ligands for the activating immune receptor NKG2D, which is expressed by NK and some T cells	DNA damaging agents
	Increase of immunogenicity of tumor cells by activating the antigen-processing machinery and altering expression patterns of antigenic peptides	paclitaxel, doxorubicin

Active immunotherapy

DNA VACCINES

DNA can be administered as naked DNA or implemented in vectors [10]. Uptake of DNA by antigen presenting cells (APC) will result in presentation in MHC I context, resulting in the induction of a Th1 response and thus the activation of cytotoxic T lymphocytes (CTL).
A major advantage of the use of DNA vaccines with respect to cellular therapies is that DNA vaccines are not patient-specific and can be generated in large amounts with high purity and they can be generated as off the shelf vaccines. In addition, the generated DNA sequence can be altered to change the intracellular processing of TAA into MHC I or MHC II context. If supplemented with bacterial sequences, these sequences can serve as adjuvants to the vaccine [10]. This type of anticancer vaccines is currently being tested in several clinical trials, but no FDA approved DNA vaccine for use in humans is available.

PEPTIDE VACCINES

Vaccination with peptides offers several advantages. Like DNA vaccines, peptide vaccines can be generated as off the shelf vaccines. In addition, vaccination can be done against well-defined peptides, which offers the possibility of avoiding vaccination with peptides that might result in undesired side-effects [11]. In addition, both CD8 T cells and CD4 T cells can theoretically be activated. However, an important limitation of the use of peptides in cancer treatment is their MHC restriction. Peptides are only suitable for patients with a certain HLA type. Moreover, most currently known peptides are MHC class I restricted. Much less information is available for peptides that are MHC II restricted. This will result in the activation of mainly $CD8^+$ T cells. Peptides binding MHC molecules of non-professional antigen presenting cells (e.g. fibroblasts) is another important drawback. On these cells, the costimulatory signals required for adequate T cell activation will not be available, rendering T cells tolerogenic.

IMMUNOSTIMULATORY CYTOKINES

Stimulating cytokines are often used prior to or following cancer treatment. They are often used in order to reconstitute the immune system after treatments with detrimental effects on the immune system, such as chemotherapy. They may also be used prior to chemotherapy in order to prevent lymphodepletion or chemotherapy-induced neutropenia. In addition, in case of leukemia, cytokines are used to mobilize latent leukemic cells prior to chemotherapy or to stimulate hematopoietic stem cells for autologous stem cell transplantation [12].
Cancer often develops in a background of chronic inflammation, which is related to the presence of high levels of pro-inflammatory cytokines. Additional cytokine treatment may thus in fact exacerbate the oncogenic potential. To date, the regulatory authorities have only approved three cytokine treatments [12]; interferon (IFN)-α2a for hairy cell leukemia and chronic myeloid leukemia, IFN-α2b in follicular lymphoma, hairy cell leukemia, AIDS-related Kaposi's sarcoma, multiple myeloma, melanoma, condyloma acuminata and cervical intraepithelial neoplasms, and interleukin (IL)-2 for metastatic melanoma and metastatic renal cell carcinoma.

ONCOLYTIC VIRUSES

Oncolytic viruses are viruses which have the capacity to directly infect and kill tumor cells. Some examples that can be used in cancer therapy are measles virus, herpes simplex virus, adenovirus and myxoma virus [13]. Like for DNA-based vaccines, described above, oncolytic viruses can be genetically engineered to obtain specific antitumor effects, such as the addition of pro-apoptotic factors. The tumor cell lysis induced by the viral particle in itself may also contribute further to the antitumor effects. The virus can cause immunogenic cell death and the release of additional TAA against which additional antitumor effects can be mounted. In addition, immunogenic factors of the viral particle, such as damage-associated molecular pattern molecules (DAMP) serve as so-called "danger signals" to alert the immune system to the presence of a virus, which could fortify the mounted immune response and result in an increased antitumor immune response [13]. Although not routinely used as anticancer treatment, several oncolytic viruses are currently being tested in advanced stages of clinical trials [13, 14].

DENDRITIC CELL-BASED VACCINES

One of the most potent cell types in the induction of an immune response is that of dendritic cells. DC-based vaccines have been used in several different setups in different cancer types. Of vital importance in DC-based vaccines is the generation of fully mature and fully functional DC with the appropriate antigens which are presented stably in MHC context. The method of generation of DC as well as the antigen loading procedure present as major challenges to this type of cancer vaccine, along with the appropriate route of administration [15]. Different methods of DC generation and antigen loading will briefly be elaborated on below.

Naturally occurring DC types

The naturally occurring DC population exists of two separate types, both originating from a different hematopoietic lineage; the conventional DC or myeloid DC, derived from the myeloid cell lineage and the plasmacytoid DC, derived from the lymphoid lineage [16]. Conventional DC consist of resident DC, which reside in the lymphoid organs and the migratory DC which reside in the peripheral organs [16].

DC generation methods

Three sources of DC have been used in clinical trials [15]. $CD34^+$ precursors, derived from blood or bone marrow, peripheral blood DC or monocyte-derived DC (moDC). The most routinely used source is that of the moDC, most importantly due to the low numbers and difficult isolation methods of $CD34^+$ precursors and peripheral blood DC. Peripheral blood monocytes can be isolated from a leukapheresis product through magnetic selection, elutriation or plastic adherence. By addition of IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF), monocytes can be differentiated into cells phenotypically and functionally resembling immature DC [17].

Antigen loading

Immature DC are capable of antigen-uptake, after which they fully mature and present the processed antigen. Once mature, DC lose the function of antigen-uptake. Consequently, *in vitro* antigen loading needs to be performed simultaneously to DC maturation. Possible antigen sources are similar to the non-DC-based immunotherapeutic options, described above. Selected, defined TAA can be used or a pool of all TAA present in the tumor [17].
Selected antigens can be loaded into DC using peptides or mRNA. When comparing these two methods, electroporation using mRNA offers several advantages over peptides. Using mRNA, the entire TAA is translated into the corresponding protein by the cytoplasmic machinery present in DC. This offers firstly the advantage that all of the peptides of the protein can be presented in MHC context, instead of selected peptides that are loaded separately. In addition, electroporation with the entire mRNA of a TAA circumvents the issue of single peptide HLA-restriction. This is an important advantage with regard to the patient population that can be targeted.
As described above for DNA vaccines, mRNA can be adapted and constructed to obtain improved results. An important modification is the addition of the DC-LAMP sequence to the mRNA sequence encoding for the TAA. The DC-LAMP signal is responsible for the shuttling of antigens that are being processed by DC to the endosomal pathway, which results in antigen presentation in MHC II context.

Consequently, coupling of DC-LAMP to mRNA will result in antigen presentation in both MHC I and MHC II context [24].

Whole tumor TAA can be introduced into DC by the use of total tumor mRNA, whole tumor cell lysates or tumor cell-DC hybrids [17]. Although quite cumbersome, the advantage of this antigen loading technique is that the whole TAA spectrum is presented to the immune system. However, this may lead to an increased possibility of autoimmune reactions.

Maturation protocols

Maturation of dendritic cells is vital to obtain fully capable DC to be used for vaccination purposes. Mature DC can be generated by adding stimuli to immature DC, such as a cytokine mixture consisting of IL-1β, tumor necrosis factor (TNF) α, currently most commonly used, or by the addition of toll-like receptor (TLR) ligands, such as LPS, poly (I:C), resiquimod and imiquimod [17, 18]. Addition of the cytokine cocktail has been shown to induce a stable, mature phenotype of DC with adequate immunostimulatory capacity [19]. Maturation using TLR ligands results in increased DC trafficking and increased priming of CD8 T cells [20-22].
These methods have been in use for many years in DC-based vaccination studies. However, a more recently developed procedure for DC maturation is the use of TriMix electroporation. TriMix is a cocktail of mRNA encoding for CD40L, constitutively active (ca)TLR4 and CD70. The combination of CD40L and caTLR4 results in CD40 ligation, providing an activation signal for immature DC. Using this protocol, like with other maturation protocols, fully mature and functional DC can be generated. In addition, through the presence of CD70, a substantial increase in T cell stimulatory capacity can be achieved [23]. This type of maturation stimulus is particularly interesting for vaccines wherein antigen loading is also performed by electroporation because antigen loading and maturation can be performed in one step.

HURDLES IN CANCER VACCINATION

As stated above, there is a lack of a consistent correlation between immunological responses and clinical responses in immunotherapy trials. The presence of immune inhibitory molecules is one of the possible reasons for this discrepancy. In order for cancer immunotherapy to be successful, a series of steps must occur successfully, recently described as the cancer immunity cycle (Fig. 2) [25].

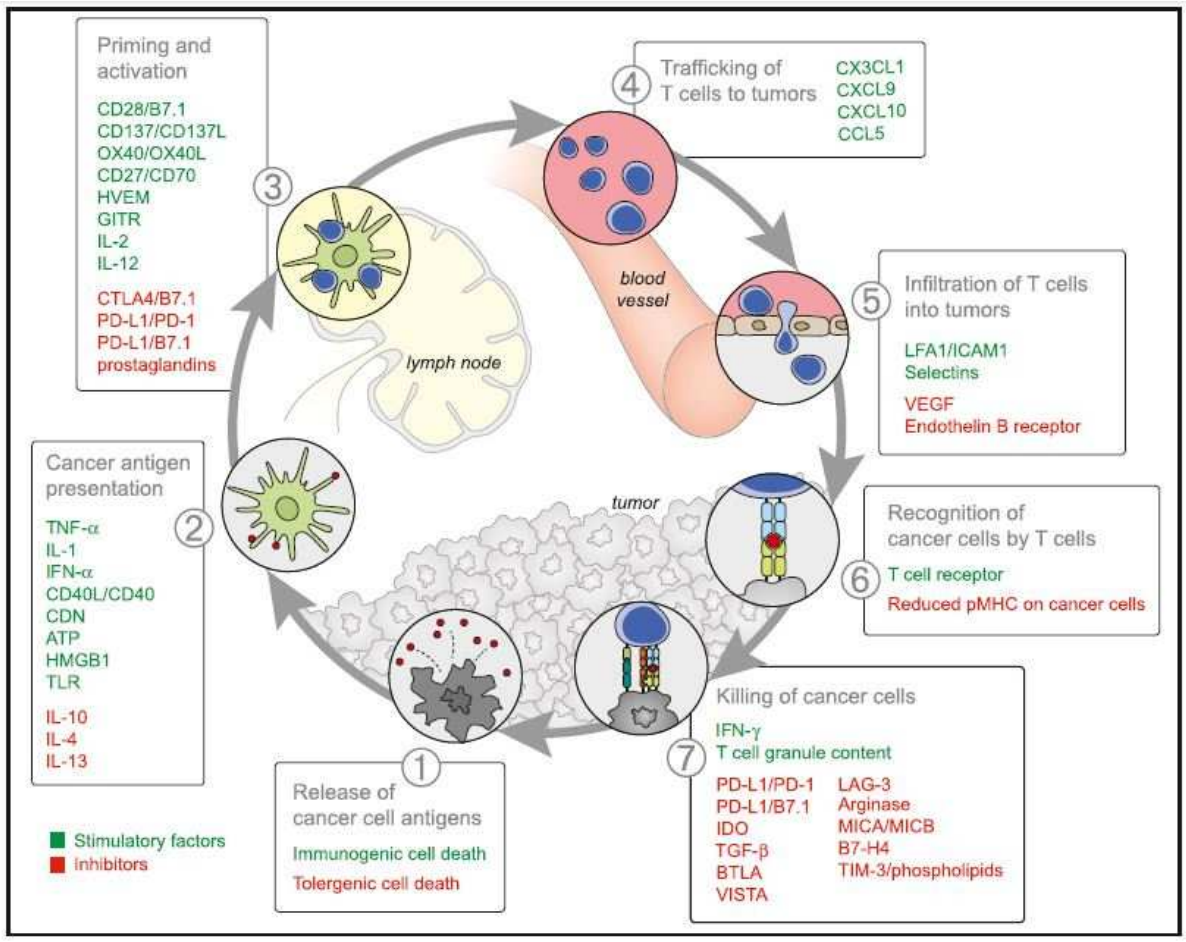


Figure 2: The cancer immunity cycle (adopted from Chen et al. [25]).

As exemplified in Fig. 2, a successful antitumor response is subject to several factors. T cells must first of all be sufficiently primed, and be capable of infiltrating the tumor and receive all necessary activation signals to become active, functional CTL. However, DC in cancer often show impaired function, which may in fact already jeopardize sufficient antigen presentation and T cell priming. Additionally, if T cell priming and infiltration are successful, tumors exploit several mechanisms to hamper T cell functioning.

The complex interplay between immunostimulatory and immune inhibitory molecules and mediators advocate for a combinatorial approach to optimize the activation of the immune system as well to intervene with immunosuppressive mechanisms and to apply these therapeutic modalities in combination with currently used standard treatments in order to improve anticancer treatment.

REFERENCES

1. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science. 2011;331(6024):1565-70.
2. Galluzzi L, Vacchelli E, Fridman WH, Galon J, Sautes-Fridman C, Tartour E, et al. Trial Watch: Monoclonal antibodies in cancer therapy. Oncoimmunology. 2012;1(1):28-37.
3. Vacchelli E, Aranda F, Eggermont A, Galon J, Sautes-Fridman C, Zitvogel L, et al. Trial Watch: Tumor-targeting monoclonal antibodies in cancer therapy. Oncoimmunology. 2014;3(1):e27048.
4. Darcy PK, Neeson P, Yong CS, Kershaw MH. Manipulating immune cells for adoptive immunotherapy of cancer. Current opinion in immunology. 2014;27C:46-52.

5. Topalian SL, Weiner GJ, Pardoll DM. Cancer immunotherapy comes of age. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011;29(36):4828-36.
6. Dudley ME, Gross CA, Langhan MM, Garcia MR, Sherry RM, Yang JC, et al. CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010;16(24):6122-31.
7. Park TS, Rosenberg SA, Morgan RA. Treating cancer with genetically engineered T cells. Trends in biotechnology. 2011;29(11):550-7.
8. Blank CU. The perspective of immunotherapy: new molecules and new mechanisms of action in immune modulation. Current opinion in oncology. 2014;26(2):204-14.
9. Shurin MR, Naiditch H, Gutkin DW, Umansky V, Shurin GV. ChemoImmunoModulation: immune regulation by the antineoplastic chemotherapeutic agents. Current medicinal chemistry. 2012;19(12):1792-803.
10. Senovilla L, Vacchelli E, Garcia P, Eggermont A, Fridman WH, Galon J, et al. Trial watch: DNA vaccines for cancer therapy. Oncoimmunology. 2013;2(4):e23803.
11. Slingluff CL, Jr. The present and future of peptide vaccines for cancer: single or multiple, long or short, alone or in combination? Cancer journal. 2011;17(5):343-50.
12. Vacchelli E, Eggermont A, Fridman WH, Galon J, Zitvogel L, Kroemer G, et al. Trial Watch: Immunostimulatory cytokines. Oncoimmunology. 2013;2(7):e24850.
13. Bauzon M, Hermiston T. Armed Therapeutic Viruses - A Disruptive Therapy on the Horizon of Cancer Immunotherapy. Frontiers in immunology. 2014;5:74.
14. Vacchelli E, Eggermont A, Sautes-Fridman C, Galon J, Zitvogel L, Kroemer G, et al. Trial watch: Oncolytic viruses for cancer therapy. Oncoimmunology. 2013;2(6):e24612.
15. Nestle FO, Farkas A, Conrad C. Dendritic-cell-based therapeutic vaccination against cancer. Current opinion in immunology. 2005;17(2):163-9.
16. Radford KJ, Tullett KM, Lahoud MH. Dendritic cells and cancer immunotherapy. Current opinion in immunology. 2014;27C:26-32.
17. Schuler G, Schuler-Thurner B, Steinman RM. The use of dendritic cells in cancer immunotherapy. Current opinion in immunology. 2003;15(2):138-47.
18. Kalinski P, Urban J, Narang R, Berk E, Wieckowski E, Muthuswamy R. Dendritic cell-based therapeutic cancer vaccines: what we have and what we need. Future oncology. 2009;5(3):379-90.
19. Jonuleit H, Kuhn U, Muller G, Steinbrink K, Paragnik L, Schmitt E, et al. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. European journal of immunology. 1997;27(12):3135-42.
20. Ahonen CL, Gibson SJ, Smith RM, Pederson LK, Lindh JM, Tomai MA, et al. Dendritic cell maturation and subsequent enhanced T-cell stimulation induced with the novel synthetic immune response modifier R-848. Cellular immunology. 1999;197(1):62-72.
21. Michiels A, Breckpot K, Corthals J, Tuyaerts S, Bonehill A, Heirman C, et al. Induction of antigen-specific CD8+ cytotoxic T cells by dendritic cells co-electroporated with a dsRNA analogue and tumor antigen mRNA. Gene therapy. 2006;13(13):1027-36.
22. Prins RM, Craft N, Bruhn KW, Khan-Farooqi H, Koya RC, Stripecke R, et al. The TLR-7 agonist, imiquimod, enhances dendritic cell survival and promotes tumor antigen-specific T cell priming: relation to central nervous system antitumor immunity. Journal of immunology. 2006;176(1):157-64.
23. Bonehill A, Tuyaerts S, Van Nuffel AM, Heirman C, Bos TJ, Fostier K, et al. Enhancing the T-cell stimulatory capacity of human dendritic cells by co-electroporation with CD40L, CD70 and constitutively active TLR4 encoding mRNA. Molecular therapy : the journal of the American Society of Gene Therapy. 2008;16(6):1170-80.
24. Bonehill A, Heirman C, Tuyaerts S, Michiels A, Breckpot K, Brasseur F, et al. Messenger RNA-electroporated dendritic cells presenting MAGE-A3 simultaneously in HLA class I and class II molecules. Journal of immunology. 2004;172(11):6649-57.
25. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39(1):1-10

Project aims

The primary aim of the project is to explore and develop an mRNA based DC vaccine for uterine tumors.

The preclinical work is subdivided in the following secondary aims:

1. Exploration of available data on the immune system in uterine cancer
2. Selection and experimental validation of existing tumor-associated antigens (TAA) to define appropriate targets for the dendritic cell vaccine.
3. Analysis of the immunosuppressive environment in uterine tumors to identify possible therapeutic targets

In addition, work was performed within the framework of 2 separate clinical studies:

1. Immune monitoring of uterine cancer patients vaccinated with a WT1 dendritic cell vaccine
2. Synchronization of treatment with low-dose cyclophosphamide treatment to CRP immune cycles in patients with ovarian or uterine cancer

Part II The immune system in the uterus

Part II

Chapter 2 The immune system in the normal uterus and its implication in endometrial cancer development

---

Vanderstraeten A, Tuyaerts S and Amant F
Submitted

The immune system in the normal endometrium and its implication in endometrial cancer development

ABSTRACT

Although described for the first time already some decades ago, the contribution of the immune system to the establishment of tumors has long not been extensively pursued. Over the last decade, however, more and more evidence is accumulating concerning the role the immune system plays in tumor development and progression and its possible role in patient prognosis. In addition, interest is growing for preclinical and clinical research concerning the use of the immune system in the treatment of cancer.
Immunotherapy for gynecological cancers in general, and especially for endometrial cancer is still in its early days. Only a small number of studies, with varying success rates, have been published.
We currently provide a concise overview of the available literature on the role of the immune system in the normal endometrium and in endometrial cancer as well as the possible implication for future immunotherapeutic studies.

INTRODUCTION

Many risk factors involved in the etiology of endometrial cancer have been described. Obesity and physical inactivity are two important risk factors for the development of uterine tumors, along with elevated blood pressure, high energy intake, high serum glucose levels and increased exposure to estrogens [1]. For some of these risk factors, effects on and interactions with the immune system are described. Hormonal fluctuations during the menstrual cycle have been described to modulate immune functions, reviewed by Wira et al. [2]. Hormonal fluctuations and interactions with immune cells result in a protective environment against invading pathogens while creating a favorable environment for embryonal implantation and fetal development. Obesity, which is related to an increased risk of developing endometrial cancer, is considered a chronic inflammatory state, causing increased release of pro-inflammatory cytokines such as IL-6 and CRP [3].
In addition to the effect of the described risk factors on the immune system, the vast majority of endometrial cancer cases is diagnosed in post-menopausal women and often in elderly patients. Age has an important influence on the immune system, the so-called immunosenescence, which parallels hormonal changes which occur with increasing age [4]. Aging causes an overall decrease in immune-related functions and results in a latent pro-inflammatory state.
Taken together, these data indicate that risk factors associated with the occurrence of endometrial cancer have an important influence on the immune system. In the current review, we provide an overview of the role the immune system plays in the normal non-pregnant uterus and how changes in the immune system may play a role in the development of uterine tumors and possible clinical outcome. This knowledge is important for successful further development of immunotherapeutic strategies for uterine cancer.

1. The uterine immune system under physiological conditions and in cancer

The immune system in the normal uterus serves a dual purpose. On the one hand, it plays a role in the protection against pathogens, while on the other hand, it has the ability to adapt to an

immunosuppressive state in order to create feto-maternal tolerance towards a semi-allogeneic fetus. These separate functions involve the complex interplay of the hormonal fluctuations of the menstrual cycle and the immune system. Normal endometrium is naturally under strict hormonal control. It is under constant control of the variations of estradiol and progesterone during the menstrual cycle. Both the innate and adaptive arm of the immune system are influenced by these hormonal changes. Several risk factors have been described for endometrial cancer, which may be linked with increased inflammation of the endometrial tissue, reviewed by Modugno et al. [5]. Increased exposure to estrogens has been shown to be associated with endometrial cancer development, due to the mitogenic effect of estrogens [6-8]. Consequently, the estrogen-related carcinogenesis may be related to inflammatory events. Chronic inflammation has been linked to cancer development [9]. Several inflammation pathways are involved in carcinogenesis. Many of these pathways are initialized by, among others, activation of STAT 3 or NF-κB [10]. The detailed role these pathways and their downstream mediators play in carcinogenesis is beyond the scope of this review and briefly summarized in Fig. 1. This interplay is discussed and further elaborated on by Elinav et al. [10].

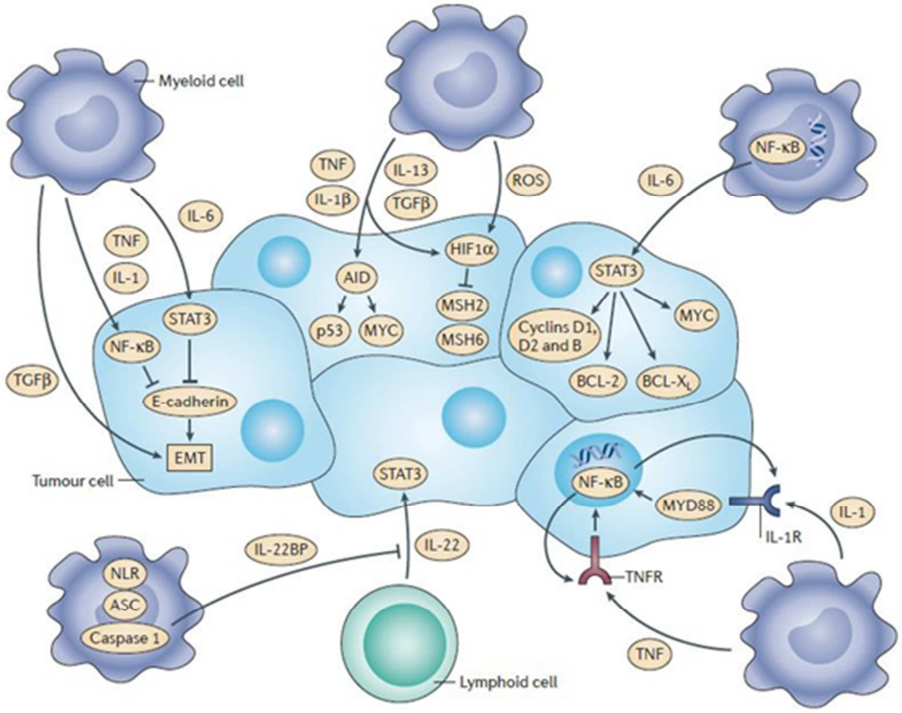


Figure 1: Inflammatory signaling and carcinogenesis.
Pro-inflammatory cytokines induce signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB) signalling in cancer cells, which leads to the suppression of apoptosis and the promotion of cell cycle progression. Inflammasome-dependent interleukin-22 binding protein (IL-22BP) secretion inhibits IL-22-driven STAT3 induction. Genomic destabilization can be promoted by cytokine-mediated ectopic expression of activation-induced cytidine deaminase (AID) and by hypoxia-dependent suppression of DNA repair mechanisms. In addition, STAT3 and NF-κB signalling also induces epithelial–mesenchymal transition (EMT) by downregulating the expression of epithelial differentiation markers. ASC, apoptosis-associated speck-like protein containing a CARD; HIF1α, hypoxia-inducible factor 1α; IL-1R, IL-1 receptor; MYD88, myeloid differentiation primary response 88; NLR, NOD-like receptor; ROS, reactive oxygen species; TGFβ, transforming growth factor-β; TNF, tumor necrosis factor; TNFR, TNF receptor. (reproduced with permission from Elinav et al. [10]).

1.1 Immune functions of normal and malignant endometrial cells

The endometrial epithelium serves as the primary line of defense against viruses and other pathogens entering the uterus. The epithelial cells form an integral part of the mucosal immune system. Next to forming a physical barrier, the epithelial cells have several direct immune-related functions, one of which is the secretion of defensins [11]. Defensins form a part of the innate immune system, considering their immediate antimicrobial function and their ability to activate the adaptive immune system. Defensins have for example been shown to attract T cells and immature dendritic cells (DC) in response to binding to the C-C chemokine receptor type 6 (CCR6) [12]. Other secreted molecules include macrophage inflammatory protein (MIP) 3α, also a ligand for CCR6, and secretory leukocyte protease inhibitor (SLPI) [13, 14]. Contradictory, uterine epithelial cells secrete unidentified, soluble immune mediators that confer a tolerogenic phenotype to DC [15].
Obesity and diabetes have also been shown to be associated with increased release of pro-inflammatory molecules, such as IL-6, TNF-α, CRP, leptin and macrophage migration inhibitory factor [3, 16]. Two studies evaluating the serum levels of IL-6, TNF-α and CRP and the risk of developing endometrial cancer, have shown that elevated levels of CRP are associated with endometrial cancer risk [17, 18]. Wang et al. found this correlation after correcting for BMI and age [18]. Friedenreich et al., in addition, found CRP and endometrial cancer risk were associated with high BMI and serum IL-6 and endometrial cancer risk to be associated with low BMI [17].
Indoleamine 2,3-dioxygenase (IDO), which is responsible for T cell suppression through the deprivation of the for T cells crucial nutrient tryptophan, is up-regulated in secretory versus proliferative endometrium. The presence of the enzyme may serve a dual protective role: it functions as an anti-bacterial agent, while on the other hand it induces suppression of T cells. The latter will create an immunosuppressive state to allow embryonal implantation [19]. IDO is also expressed by endometrial carcinoma cells [20-22], and was proven to be associated with myometrial invasion, lymph node metastases and lymphovascular space involvement [21]. In addition, high IDO expression correlated with decreased $CD8^+$ TIL and NK cell involvement and was associated with poor survival [20]. Thus, both in normal and malignant endometrium, IDO's primary function seems to be induction of immunosuppression in order to allow embryonal implantation or tumor growth.
Endometrial epithelial cells are also potent antigen-presenting cells. Ferguson et al. found expression of MHC class I in endometrial glands as well as stromal cells and endothelial cells. MHC class II, on the contrary, was found to be expressed in the endometrial glands in approximately 50% of normal endometrium samples [23]. Fahey et al. have shown that cultured epithelial cells express CD40 and CD1d and that epithelial cells as well as stromal endometrial cells can elicit tetanus toxoid specific T cell responses [24, 25]. In endometrial tumors, classical MHC class I was down-regulated in 48.5% of 520 tumors, which is associated with worse disease prognosis [26]. In addition, the non-classical MHC class I molecule, HLA-G, was up-regulated in 39.8% of samples, corroborating results of Barrier et al., who found expression of HLA-G in 55% of samples [27]. Although requiring further investigation, the up-regulation of HLA-G molecules in endometrial tumors may be a protective mechanism to avoid NK cell lysis in case of down-regulation of MHC class I molecules, as shown in other tumors [28, 29]. MHC class II was found to be present in only a small portion of malignant endometrial cells. Low presence of both the classical MHC I and II molecules as well as the up-regulation of the non-classical HLA-G point to a poor antigen-presenting capacity of endometrial tumor cells [30, 31]. Cells in the underlying stroma, however, do show MHC II positivity [30]. Taken together, normal endometrial cells can present antigens in the context of MHC molecules, probably as a defense mechanism to

pathogens. Endometrial tumor cells however, down-regulate expression of MHC molecules to mediate immune escape.

Lastly, several members of the B7-H family have been described. We recently described the presence of these molecules both in normal endometrium and uterine tumors [22]. We found expression of PD-L1 (B7-H1), and B7-H4 in the vast majority of normal endometria, while PD-L2 (B7-DC) was present in approximately half of normal endometria, albeit at low levels. All of these molecules were also present in endometrial cancer [22]. When comparing the expression levels of all molecules, we did not find an up-regulation in endometrial tumors. Although the investigated population was fairly small for sound analysis, a trend towards decreased survival was found in PD-L1^{+} tumors [22]. Our results on B7-H4 are contradictory a previously published study, in which B7-H4 was reported to be significantly up-regulated in endometrial tumors [32, 33]. The expression pattern of this molecule was mainly cytoplasmic in conjunction with strong membrane staining and has been shown to negatively correlate with the number of TIL, both the T cell population as a whole (CD3^{+}) and the separate CTL population (CD8^{+}) [32]. For the latter, this correlation was also found for B7-H3 [34].These data indicate that, since for most of these mediators no up-regulation was found in endometrial tumors, these molecules may also exert their immunosuppressive functions in both the normal and cancerous situation, as described above for IDO.

Table 1: Overview of immunological mediators in uterine tumors.

Molecule/cell type	**Normal endometrium**	**Uterine tumors**		
		Available data	Correlation to clinicopathology	Relation to prognosis
MHC class I	Expressed	Down-regulated	Down-regulated in advanced and undifferentiated tumors	Worse prognosis
MHC class II	Expressed in ~ 50% of cases	Present in minority of tumor cells		
HLA-G	Conflicting data	Up-regulated		
IDO	Up-regulated in secretory phase	Up-regulated	Associated with myometrial invasion, lymph node metastases and lymphovascular space involvement	Associated with poor survival
NK cells	Increase during menstrual cycle	Low levels, increased upon progestin treatment	Activity decreased in advanced disease	
Macrophages	Increase during menstrual cycle	Location-dependent pro- or anti-tumor effects	Location-dependent	Location-dependent
Neutrophils	Increase during menstrual cycle	Increased	Increased NLR associated with lymph node metastasis	
DC	Low levels	Increased	Negatively correlated with the clinical stage and lymph node metastasis	
B cells	Present in aggregates			
T cells	Present in aggregates	Conflicting data		Dependent on location and phenotype
Treg	Increase during menstrual cycle	Increased compared to blood	Increased in advanced disease	Worse prognosis
MDSC	Unknown	Present with higher frequency of granulocytic subtype		Unknown

NLR: neutrophil to lymphocyte ratio.

1.2 Infiltration by immune cells

Next to the mediators just discussed, which can attract immune cells, immune cells themselves are present as part of endometrial tissue. The exact nature of the immune cells present in endometrial tissue/tumors and their function will be elaborated on below. A schematic representation of the presence of immune cells in normal endometrium is given in Fig. 2a and the main players in the tumor microenvironment are depicted in Fig. 2b. Table 1 gives a concise overview of the immunological players in endometrium and endometrial tumors and their implication in tumor biology.

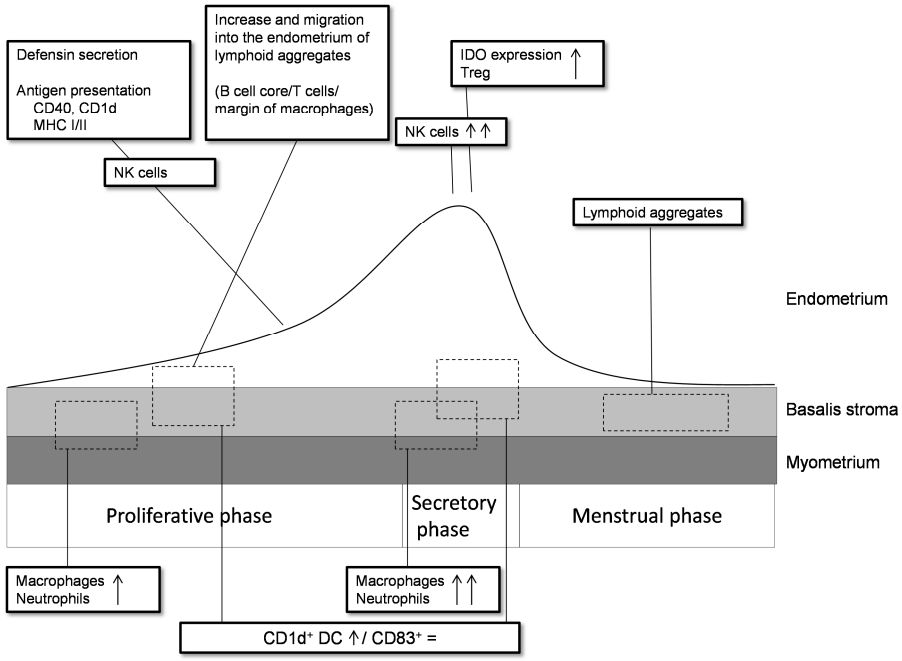
1.2.1 Innate immune cells

Macrophages represent approximately 10% of the total cellular population of the endometrium. They are mostly present in the endometrial stroma and myometrial connective tissue [35]. Their frequency is the highest prior to menstruation, along with the frequency of neutrophils. The latter play a role in the breakdown of the endometrial tissue at menstruation as well as in the elevation of immune protection during the disruption of the protective barrier of the endometrial epithelium [36]. Macrophages play a paradoxical role in cancer, in a sense that they can have both a pro- and anti-tumorigenic function [37]. Tumor-associated macrophages (TAM) located in the focal necrotic center of the tumor and TAM at the tumor margin correlated with disease progression and with clinicopathological features of the tumor [37, 38]. TAM at the tumor margin were associated with the formation of lymph node metastases, indicating tumor progression, whereas macrophages in the tumor nest, the bulky area of the tumor surrounding the tumor center, were associated with a better relapse-free survival. This may be explained by local factors within the tumor, exerting different functions on macrophages. The tumor center, for example, is known to be hypoxic. This is suggested to trigger the angiogenic capacities of macrophages, leading to renewed oxygen supply and tumor progression [37]. Another cell type, like macrophages derived from the myeloid lineage, are myeloid-derived suppressor cells (MDSC). To date, to our knowledge, MDSC have only been described in endometrial cancer by our own group [22]. MDSC analysis was subdivided into the presence of monocytic MDSC ($lin^-HLA\text{-}DR^{-/lo}CD11b^+CD14^+$) and granulocytic MDSC ($lin^-HLA\text{-}DR^{-/lo}CD11b^+CD14^-$). Both MDSC of the monocytic and granulocytic type were found, although the majority of the identified population was of the granulocytic type. This subtype has been described to have the strongest suppressive capacity compared to the monocytic subtype [39], providing evidence of increased immunosuppression in endometrial tumors.

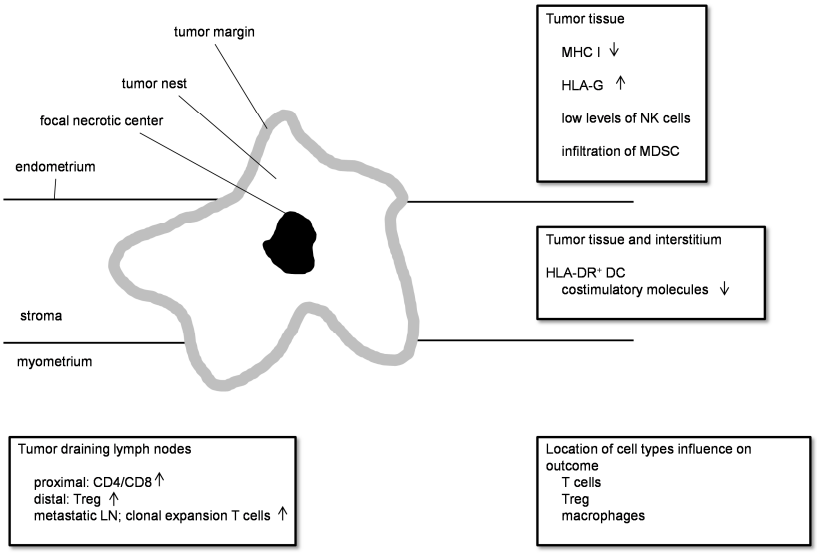
The largest representative of the innate immune system however, is that of natural killer cells (NK cells). Like for the cells described above, their numbers in normal endometrium vary depending on the phase in the cycle. The highest number of NK cells is found in the secretory phase of the cycle. At this point NK cells represent about 70% of the total leukocyte population [35]. This is likely the result of both increased IL-15 levels in the endometrium in the secretory phase and of an increased NK cell influx from peripheral blood [19]. However, research by Manaster et al. showed that the percentage of NK cells remains relatively constant at approximately 30% of the total lymphocyte population [40]. Male et al found the presence of precursor NK cells, so-called stage 3 NK cells in uterine mucosa as well as mature, stage 4, NK cells. [41]. The authors postulate that stage 3 NK cells ($CD34^-CD117^+CD94^+$) migrate into the uterus where they mature to obtain their distinct phenotype ($CD34^-CD117^{-/+}CD94^+$) [41]. Uterine NK cells are different from their counterparts in blood [42]. Like

NK cells in blood they express CD94, CD56 and CD9, but do not express CD16, CD8 or CD57. In addition, CD56 is expressed at about ten-fold higher levels in uterine NK cells than in blood NK cells [42]. Little has been described concerning the functional differences of peripheral blood NK cells and uterine NK cells. NK cells in both proliferative and secretory phase endometrium have been shown to be inert cells which lack both their cytotoxic capacity and their ability to secrete cytokines. However this can be reverted when the cells are cultured in the presence of IL-15 [40]. Stimulation with IL-15 resulted in the up-regulation of the activating NK cell receptors NKp30 and NKp44, but no difference was found in expression of NKp46 and NKG2D. In addition, IL-15 activated endometrial NK cells showed increased *in vitro* cytotoxic capability and secreted IP-10 (CXCL-10) and IFN-γ [40]. Uterine NK cells are thus suggested to be inert lymphocytes without the cytotoxic capabilities of peripheral NK cells. These NK cells are inactive during the normal menstrual cycle and are suggested to mature to fully functional NK cells during pregnancy [40]. There are only few studies focusing on NK cells in endometrial carcinoma patients. NK cell activity in peripheral blood against K562 cells was found to decrease with an increase in histological differentiation grade and myometrial invasion in early stage (stage I) endometrial carcinoma [43]. A study already published in 1987 by Timonen et al. in 8 endometrial cancer patients and 1 endometrial stromal sarcoma patient showed that unstimulated peripheral blood lymphocytes show cytotoxic responses against autologous tumor and against HeLa cells in 7/9 patients [44]. This activity was increased upon addition of recombinant IL-2. The IL-2 activated lytic precursor cells belonging to the subpopulation of lymphocytes which includes NK cells [44]. Ferguson et al. found that NK cells were virtually absent in endometrial tumors [23]. Intratumoral NK cells were analyzed immunohistochemically in endometrial carcinoma patients following progestin treatment [45]. After treatment with progestin, the total cytotoxic (granzyme B^+) lymphocyte population in the tumors increased 6.5-fold. While $CD56^+$ NK cells were low or absent pre-treatment, the NK cell frequency rose to 76% of the total cytotoxic (granzyme B^+) cell population in endometrial lesions which showed signs of regression. On the contrary, in lesions of stable or progressive nature, no increase in NK cells was noted. $CD8^+$ CTL showed a mild increase in regressing lesions, while they remained approximately constant in stable or progressive lesions. Thus, progestin treatment can attract NK cells into uterine tumors which is associated with disease improvement. In addition, these findings can explain the increased level of NK cells in the secretory phase of normal endometrium, when progesterone levels are highest.

Dendritic cells (DC) were also described in the human endometrium, at relatively low levels compared to other immune cells [46]. Throughout the cycle, these cells reside both in the functional and basal layers. The frequency of immature $CD1a^+$ DC increases during the cycle, while the mature $CD83^+$ DC population remains relatively constant. This indicates that, in accordance with their natural function, mature dendritic cells migrate from their resident tissue. In uterine tumors, HLA-DR^+ DC have been shown to be present in both the glandular cells and in interstitial tissue [47]. The functional capacity of tumor-infiltrating DC, however, has been shown to be compromised in uterine tumors, due to significantly reduced expression of the costimulatory molecules CD86, CD80 and CD40 compared to DC in normal endometrium [48].



A



B

Figure 2: The immune system in the normal uterus and in uterine cancer.

A. Fluctuations in the immune system in the normal menstrual cycle. During the proliferative phase up until the secretory phase, NK cells and macrophages proliferate and the lymphoid aggregates increase in size. The increased frequencies corroborate their function in the breakdown of the endometrium. $CD1d^+$ DC increase in frequency, while $CD83^+$ DC remain constant, possibly indicating DC migration. During the secretory phase, both IDO and the frequency of Treg are increased in order to create an immunosuppressive environment for possible embyonal implantation. Dashed rectangles indicate the location of the endometrium the cells reside in.

B. The immune system in endometrial cancer.

In contrast to in normal endometrium, MHC class I/II molecules are down-regulated, facilitating immune escape, while HLA-G is up-regulated. In addition, MDSC infiltrates are described in uterine tumors and DC have been shown to downregulate costimulatory molecules.

For T cells (incl Treg), as well as macrophages, their exact function and the resulting effect on outcome is dependent on the location within the tumor/ at the tumor margin.

1.2.2 Adaptive immune cells

T and B cells, both members of the adaptive immune system, can also be found in the normal endometrium. They are present in uterine mucosa as unique aggregates consisting of a B cell core surrounded by T cells. Additionally, these structures are surrounded by a capsule of macrophages and monocytes [49]. These structures have been suggested to be similar to the mucosa-associated lymphoid tissue (MALT), which can be found in the gastrointestinal system [50]. The T cells present in these aggregates are almost exclusively $CD8^+$ $CD45RO^+$, indicating that they are memory type effector cells [51]. These aggregates have been shown to increase in size from the proliferative phase, at this point without the B cell core, until the secretory phase of the menstrual cycle. In addition, they are absent in the menopause, indicating that their expansion is hormone-driven [49]. This is further exemplified by the observation that T cells within the aggregate express estrogen receptors [52]. Additionally, Yeaman et al. have shown that the accumulation of T cells is the result of T cell migration towards the endometrium, rather than a proliferation of single resident T cell clones [51].
The function of these aggregates is largely unknown. However, they may serve a purpose in both the creation of an immunosuppressive environment to allow feto-maternal tolerance on the one hand and on the other hand to create a protective environment against pathogens during menstruation, when the epithelial barrier is disrupted. The former is exemplified by the observation that the cytotoxic T lymphocytes in the proliferative phase of the cycle have cytotoxic capacity, while this function is severely dampened in the secretory phase, during which conception can occur [53]. In addition, $CD8^+$ T cells are still capable of exerting their full cytotoxic function, further indicating that during the secretory phase of the menstrual cycle, a temporary state of immunosuppression occurs, in order to allow possible embryonal implantation. The difference in cytotoxic capacity of T cells during the different phases of the menstrual cycle is subject to hormonal control in order to maintain the balance between immune protection and tolerance [53]. The latter function of the lymphoid aggregates is supported by the location from which they originate. During the proliferative phase, the aggregates expand from within the basalis stroma, the inner third of the endometrium that is not shed during menstruation. Consequently, the lymphoid aggregates may provide immune protection against pathogens during menstruation. Alternatively, the presence of these aggregates in the basalis stroma may be a means to prevent loss of T and B cells during menstruation [49]. This type of lymphoid structures, recently termed tertiary lymphoid structures, resemble the MALT found in the gastrointestinal system, as mentioned above. These structures have been described in several tumor types, such as colorectal cancer, lung cancer, melanoma, ovarian cancer, renal cell cancer and breast cancer [54]. The co-localization of both T and B lymphocytes in these aggregates has been shown to correlate with improved patient survival [55]. In uterine tumors, MALT-like structures have not been described to date, but tumor infiltrating lymphocytes (TIL) have been shown in different studies. Chang et al. found that $CD8^+$ (TIL) showed less expression of granzyme B and perforin than their blood counterparts, indicating possible functional defects or tumor-induced suppression [56]. However, *in vitro* activation of TIL resulted in adequate activation of TIL and induction to the same polarization profile as found in peripheral blood (i.e. main polarization to Th1 type cells). TIL have been associated with prognosis in endometrial cancer, with contradictory reports. The prognostic value of this infiltrate depends on the location within the tumor. Increased numbers of TIL, of unspecified composition, at the invasive margin of the tumor (i.e. the tumor-myometrial junction) did not have a beneficial effect on patient survival according to a study by Silverberg et al. [57]. These results were contradicted by Kondratiev et al., who found that, although confirming the presence of

$CD8^+$ TIL at the tumor invasive margin, the presence of these TIL was associated with improved prognosis [58]. However, the latter study only considered $CD8^+$ TIL at the invasive border while Silverberg et al. considered the total lymphocyte population, which may clarify these different findings. Two additional studies studied the total lymphocyte population at the invasive margin [59, 60]. Deligdisch et al observed that the presence of an infiltrate consisting of lymphocytes and plasma cells, potentially indicating the described tertiary lymphoid structures, appeared to be related to low-grade endometrial tumors, and suggested that TIL are associated with a favorable prognosis [60]. A later study by Ambros et al. refuted this suggested association [59]. Intratumoral $CD8^+$ TIL have been associated with improved disease-free survival in both type I and type II endometrial cancer [61]. These intratumoral TIL were found more frequently in low grade tumors than in high grade tumors. The presence of $CD45RO^+$ T cells, indicating memory T cells, was also shown. Moreover, the presence of memory T cells was associated with increased overall survival and with reduced events of recurrence [61]. Chang et al. described that the majority of tumor infiltrating $CD8^+$ T cells are $CD28^-CD45RA^-CD45RO^+$, defining terminally differentiated T cells. In addition, the T cells appeared to be in an activated state, exemplified by the expression of CD69, CD103 and CD152 [56]. In the proximal tumor draining lymph nodes (TDLN), the CD4/CD8 ratio is increased [62]. In addition, Yamamoto et al. found that clonally expanded T cells are absent from TDLN in patients with local endometrial tumors, while clonally expanded T cells could be retrieved from TDLN and peripheral blood in patients suffering from metastatic cancer, supporting the role of immune responses to solid tumors [63]. This specific appearance of T cell clones in TDLN of metastatic tumors may be a consequence of direct T cell priming by (metastasized) tumor cells present in the TDLN in metastatic tumors. This results in the expansion of T cell clones in affected lymph nodes. This direct priming does not occur in unaffected lymph nodes, as is the case in early-stage disease [64]. The results of Yamamoto et al. expand earlier findings of Garzetti et al. [65], who did not find any clinical significance in the lymphocyte distribution in lymph nodes in patients with early stage disease. In addition, Garzetti et al. showed that myometrial invasion with or without lymphovascular space involvement was associated with increased $CD16^+$ and $CD56^+$ cells, defining NK cells, in pelvic nodes [65].

Regulatory T cells (Treg) have a natural function to suppress ongoing immune responses when they are no longer necessary. However, this suppressive property of these cells may also cause suppression of an antitumor immune response. Treg have been shown to be increased in peripheral blood of normal controls in the late follicular phase [66]. Analysis of the Treg frequency in the endometrium showed that Treg are present in only low frequencies in the endometrium and that the frequency is higher in the proliferative phase compared to the secretory phase [67]. Collectively, these data indicate that the frequency of Treg cells appears to increase during the proliferative phase and is reduced after ovulation.

Several studies have reported the presence of regulatory T cells (Treg) in endometrial carcinoma. Intratumoral $CD4^+CD25^+$ Treg, expressing higher levels of FoxP3, CD103 and GITR, are increased compared to peripheral blood [56]. In this particular study, it was also shown that, like CTL, intratumoral Treg also express granzyme B, indicating the capacity to lyse effector cells. However, Treg in stromal tissue were found to be significantly lower in tumor samples compared to normal endometrium [68]. Although lower, high Treg counts in tumor samples were shown to correlate with increased vascularity [68], tumor grade, stage, the extent of lymph node metastases and myometrial

invasion [56] as well as worse disease-free survival [69]. The latter has also been shown to result from the presence of high Treg/CD8 and Treg/CD4 ratios [69]. In distal TDLN, the proportion of functional regulatory T cells is increased [62].

Taken together, the fluctuations of the different cell types during the normal menstrual cycle are a further indication of the dual role the immune system plays in the uterus, described earlier. The immunosuppressive capacity that certain cell types, such as Treg, have in the framework of feto-maternal tolerance, also contributes to immune escape. Some cell types, such as macrophages and T cells appear to have a different effect on the outcome of a tumor, depending on the location at which they reside. This likely indicates that at different sites within the tumor or the tumor microenvironment the immune system may be differentially influenced in such a way that the functional capacities of the immune cells are influenced towards either an anti-tumor or a pro-tumor profile.

2. Clinical implications

The currently reviewed data provide some insight into several immune mechanisms in uterine tumors and indicate possible options for therapeutic modalities. The composition of the intratumoral immune infiltrate may have an important influence on treatment outcome. This phenomenon has recently been described in ovarian cancer [70]. It was shown that the 5-year survival rate of ovarian cancer patients who underwent debulking surgery and received adjuvant chemotherapy was at least 6 times higher in patients with an intratumoral T cell infiltrate in the tumor islet, compared to patients without T cell infiltrate [70]. Several negative immune regulators are present and possibly active in endometrial cancer. Of the currently reviewed regulators, several could present as valuable targets for therapeutic intervention. Firstly, IDO and PD-L2 are useful targets, although only in a limited percentage of tumors [22]. Several trials are currently ongoing to evaluate the use of IDO inhibitor 1-methyltryptophan (registered at www.clinicaltrials.gov). No studies were listed to evaluate the use of an IDO inhibitor in endometrial cancer. Several trials are currently ongoing to evaluate its use, whether or not in combination with other treatments in ovarian cancer and peritoneal tumors. Both PD-L1 and B7-H4 could represent targets in EMCAR, considering their high expression levels. Antibodies directed against PD-L1 or the receptor PD-1 are being used in trials for several solid tumors. Anti PD-L1 treatment resulted in objective response rates ranging from 6 to 17% in patients with solid tumors, among which melanoma, renal cell carcinoma and non-small cell lung cancer, while anti-PD-1 led to objective response rates up to 27% [71, 72]. MDSC also represent a valuable target in endometrial cancer. Preliminary data of a clinical trial evaluating MDSC targeting with the use of all trans retinoic acid (ATRA) showed promising results. In small cell lung cancer patients, co-treatment with a DC vaccine and ATRA resulted in a substantial increase in immune response after vaccination, as exemplified by an increase in IFN-γ-secreting antigen-specific T cells [73].

Lastly, the presence of NK cells in uterine tumors is correlated with a beneficial treatment outcome in uterine tumors. This provides motivation for the use of adoptive NK cell therapy in uterine tumors. This type of therapy still remains to be explored for these tumors.

CONCLUSION

The currently outlined data clearly show that the immune system is present and active both in normal endometrium as well as in endometrial tumors. In the normal endometrium, the immune system plays a central role in the protection against pathogens and in safeguarding feto-maternal tolerance. Like this dual role in the healthy situation, it also has both a pro- and anti-tumorigenic function. In our opinion, the interplay between positive and negative players and mechanisms in tumor development and progression, provides possible intervention options in the treatment of endometrial cancer, which deserves further attention in future research.

REFERENCES

1. Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E, Vergote I. Endometrial cancer. Lancet. 2005;366(9484):491-505.
2. Wira CR, Fahey JV, Ghosh M, Patel MV, Hickey DK, Ochiel DO. Sex hormone regulation of innate immunity in the female reproductive tract: the role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens. American journal of reproductive immunology. 2010;63(6):544-65.
3. Visser M, Bouter LM, McQuillan GM, Wener MH, Harris TB. Elevated C-reactive protein levels in overweight and obese adults. JAMA : the journal of the American Medical Association. 1999;282(22):2131-5.
4. Pfister G, Savino W. Can the immune system still be efficient in the elderly? An immunological and immunoendocrine therapeutic perspective. Neuroimmunomodulation. 2008;15(4-6):351-64.
5. Modugno F, Ness RB, Chen C, Weiss NS. Inflammation and endometrial cancer: a hypothesis. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2005;14(12):2840-7.
6. Austin H, Austin JM, Jr., Partridge EE, Hatch KD, Shingleton HM. Endometrial cancer, obesity, and body fat distribution. Cancer research. 1991;51(2):568-72.
7. Potischman N, Hoover RN, Brinton LA, Siiteri P, Dorgan JF, Swanson CA, et al. Case-control study of endogenous steroid hormones and endometrial cancer. Journal of the National Cancer Institute. 1996;88(16):1127-35.
8. Zeleniuch-Jacquotte A, Akhmedkhanov A, Kato I, Koenig KL, Shore RE, Kim MY, et al. Postmenopausal endogenous oestrogens and risk of endometrial cancer: results of a prospective study. British journal of cancer. 2001;84(7):975-81.
9. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
10. Elinav E, Nowarski R, Thaiss CA, Hu B, Jin C, Flavell RA. Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. Nature reviews Cancer. 2013;13(11):759-71.
11. Wira CR, Grant-Tschudy KS, Crane-Godreau MA. Epithelial cells in the female reproductive tract: a central role as sentinels of immune protection. American journal of reproductive immunology. 2005;53(2):65-76.
12. Yang D, Chertov O, Bykovskaia SN, Chen Q, Buffo MJ, Shogan J, et al. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science. 1999;286(5439):525-8.
13. Fahey JV, Wira CR. Effect of menstrual status on antibacterial activity and secretory leukocyte protease inhibitor production by human uterine epithelial cells in culture. The Journal of infectious diseases. 2002;185(11):1606-13.
14. Fahey JV, Schaefer TM, Wira CR. Sex hormone modulation of human uterine epithelial cell immune responses. Integrative and comparative biology. 2006;46(6):1082-7.

15. Ochiel DO, Ghosh M, Fahey JV, Guyre PM, Wira CR. Human uterine epithelial cell secretions regulate dendritic cell differentiation and responses to TLR ligands. Journal of leukocyte biology. 2010;88(3):435-44.
16. Dandona P, Aljada A, Ghanim H, Mohanty P, Tripathy C, Hofmeyer D, et al. Increased plasma concentration of macrophage migration inhibitory factor (MIF) and MIF mRNA in mononuclear cells in the obese and the suppressive action of metformin. The Journal of clinical endocrinology and metabolism. 2004;89(10):5043-7.
17. Friedenreich CM, Langley AR, Speidel TP, Lau DC, Courneya KS, Csizmadi I, et al. Case-control study of inflammatory markers and the risk of endometrial cancer. European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation. 2013;22(4):374-9.
18. Wang T, Rohan TE, Gunter MJ, Xue X, Wactawski-Wende J, Rajpathak SN, et al. A prospective study of inflammation markers and endometrial cancer risk in postmenopausal hormone nonusers. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2011;20(5):971-7.
19. Lobo SC, Huang ST, Germeyer A, Dosiou C, Vo KC, Tulac S, et al. The immune environment in human endometrium during the window of implantation. American journal of reproductive immunology. 2004;52(4):244-51.
20. de Jong RA, Kema IP, Boerma A, Boezen HM, van der Want JJ, Gooden MJ, et al. Prognostic role of indoleamine 2,3-dioxygenase in endometrial carcinoma. Gynecologic oncology. 2012;126(3):474-80.
21. Ino K, Yamamoto E, Shibata K, Kajiyama H, Yoshida N, Terauchi M, et al. Inverse correlation between tumoral indoleamine 2,3-dioxygenase expression and tumor-infiltrating lymphocytes in endometrial cancer: its association with disease progression and survival. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(8):2310-7.
22. Vanderstraeten A, Luyten C, Verbist G, Tuyaerts S, Amant F. Mapping the immunosuppressive environment in uterine tumors: implications for immunotherapy. Cancer immunology, immunotherapy : CII. 2014;63(6):545-57.
23. Ferguson A, Moore M, Fox H. Expression of MHC products and leucocyte differentiation antigens in gynaecological neoplasms: an immunohistological analysis of the tumour cells and infiltrating leucocytes. British journal of cancer. 1985;52(4):551-63.
24. Fahey JV, Wallace PK, Johnson K, Guyre PM, Wira CR. Antigen presentation by human uterine epithelial cells to autologous T cells. American journal of reproductive immunology. 2006;55(1):1-11.
25. Wallace PK, Yeaman GR, Johnson K, Collins JE, Guyre PM, Wira CR. MHC class II expression and antigen presentation by human endometrial cells. The Journal of steroid biochemistry and molecular biology. 2001;76(1-5):203-11.
26. Bijen CB, Bantema-Joppe EJ, de Jong RA, Leffers N, Mourits MJ, Eggink HF, et al. The prognostic role of classical and nonclassical MHC class I expression in endometrial cancer. International journal of cancer Journal international du cancer. 2010;126(6):1417-27.
27. Barrier BF, Kendall BS, Sharpe-Timms KL, Kost ER. Characterization of human leukocyte antigen-G (HLA-G) expression in endometrial adenocarcinoma. Gynecologic oncology. 2006;103(1):25-30.
28. Ibrahim EC, Guerra N, Lacombe MJ, Angevin E, Chouaib S, Carosella ED, et al. Tumor-specific up-regulation of the nonclassical class I HLA-G antigen expression in renal carcinoma. Cancer research. 2001;61(18):6838-45.
29. Paul P, Rouas-Freiss N, Khalil-Daher I, Moreau P, Riteau B, Le Gal FA, et al. HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(8):4510-5.
30. Lazaris A, Chatzigianni EB, Xidias G, Panoskaltsis TA, Thomopoulou GC, Eftychiadis CA, et al. Tissue evaluation of immune markers in endometrial and cervical carcinomas. Journal of experimental & clinical cancer research : CR. 2004;23(2):269-75.

31. Tamiolakis D, Venizelos J, Lambropoulou M, Nikolaidou S, Tsikouras P, Jivannakis T, et al. Local immune response in serous papillary carcinoma of the endometrium. Histology and histopathology. 2005;20(2):403-8.
32. Miyatake T, Tringler B, Liu W, Liu SH, Papkoff J, Enomoto T, et al. B7-H4 (DD-O110) is overexpressed in high risk uterine endometrioid adenocarcinomas and inversely correlated with tumor T-cell infiltration. Gynecologic oncology. 2007;106(1):119-27.
33. Qian Y, Shen L, Cheng L, Wu Z, Yao H. B7-H4 expression in various tumors determined using a novel developed monoclonal antibody. Clinical and experimental medicine. 2011;11(3):163-70.
34. Brunner A, Hinterholzer S, Riss P, Heinze G, Brustmann H. Immunoexpression of B7-H3 in endometrial cancer: relation to tumor T-cell infiltration and prognosis. Gynecologic oncology. 2012;124(1):105-11.
35. Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L. Innate and adaptive immunity in female genital tract: cellular responses and interactions. Immunological reviews. 2005;206:306-35.
36. Hickey DK, Patel MV, Fahey JV, Wira CR. Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. Journal of reproductive immunology. 2011;88(2):185-94.
37. Ohno S, Ohno Y, Suzuki N, Kamei T, Koike K, Inagawa H, et al. Correlation of histological localization of tumor-associated macrophages with clinicopathological features in endometrial cancer. Anticancer research. 2004;24(5C):3335-42.
38. Soeda S, Nakamura N, Ozeki T, Nishiyama H, Hojo H, Yamada H, et al. Tumor-associated macrophages correlate with vascular space invasion and myometrial invasion in endometrial carcinoma. Gynecologic oncology. 2008;109(1):122-8.
39. Raber PL, Thevenot P, Sierra R, Wyczechowska D, Halle D, Ramirez ME, et al. Subpopulations of Myeloid-Derived Suppressor Cells (MDSC) impair T cell responses through independent nitric oxide-related pathways. International journal of cancer Journal international du cancer. 2013.
40. Manaster I, Mizrahi S, Goldman-Wohl D, Sela HY, Stern-Ginossar N, Lankry D, et al. Endometrial NK cells are special immature cells that await pregnancy. Journal of immunology. 2008;181(3):1869-76.
41. Male V, Hughes T, McClory S, Colucci F, Caligiuri MA, Moffett A. Immature NK cells, capable of producing IL-22, are present in human uterine mucosa. Journal of immunology. 2010;185(7):3913-8.
42. Yang Z, Kong B, Mosser DM, Zhang X. TLRs, macrophages, and NK cells: our understandings of their functions in uterus and ovary. International immunopharmacology. 2011;11(10):1442-50.
43. Garzetti GG, Ciavattini A, Goteri G, Tranquilli AL, Muzzioli M, Fabris N, et al. Natural killer cell activity in stage I endometrial carcinoma: correlation with nuclear grading, myometrial invasion, and immunoreactivity of proliferating cell nuclear antigen. Gynecologic oncology. 1994;55(1):111-4.
44. Timonen T, Lehtovirta P, Saksela E. Interleukin-2-stimulated natural killer activity against malignant and benign endometrium. International journal of cancer Journal international du cancer. 1987;40(4):479-83.
45. Witkiewicz AK, McConnell T, Potoczek M, Emmons RV, Kurman RJ. Increased natural killer cells and decreased regulatory T cells are seen in complex atypical endometrial hyperplasia and well-differentiated carcinoma treated with progestins. Human pathology. 2010;41(1):26-32.
46. Schulke L, Manconi F, Markham R, Fraser IS. Endometrial dendritic cell populations during the normal menstrual cycle. Human reproduction. 2008;23(7):1574-80.
47. Lijun Z, Xin Z, Danhua S, Xiaoping L, Jianliu W, Huilan W, et al. Tumor-infiltrating dendritic cells may be used as clinicopathologic prognostic factors in endometrial carcinoma. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society. 2012;22(5):836-41.
48. Jia J, Wang Z, Li X, Wang Z, Wang X. Morphological characteristics and co-stimulatory molecule (CD80, CD86, CD40) expression in tumor infiltrating dendritic cells in human endometrioid

adenocarcinoma. European journal of obstetrics, gynecology, and reproductive biology. 2012;160(2):223-7.
49. Yeaman GR, Guyre PM, Fanger MW, Collins JE, White HD, Rathbun W, et al. Unique CD8+ T cell-rich lymphoid aggregates in human uterine endometrium. Journal of leukocyte biology. 1997;61(4):427-35.
50. Marshall RJ, Jones DB. An immunohistochemical study of lymphoid tissue in human endometrium. International journal of gynecological pathology : official journal of the International Society of Gynecological Pathologists. 1988;7(3):225-35.
51. Yeaman GR, Collins JE, Fanger MW, Wira CR, Lydyard PM. CD8+ T cells in human uterine endometrial lymphoid aggregates: evidence for accumulation of cells by trafficking. Immunology. 2001;102(4):434-40.
52. Tabibzadeh SS, Satyaswaroop PG. Sex steroid receptors in lymphoid cells of human endometrium. American journal of clinical pathology. 1989;91(6):656-63.
53. White HD, Crassi KM, Givan AL, Stern JE, Gonzalez JL, Memoli VA, et al. CD3+ CD8+ CTL activity within the human female reproductive tract: influence of stage of the menstrual cycle and menopause. Journal of immunology. 1997;158(6):3017-27.
54. Goc J, Fridman WH, Sautes-Fridman C, Dieu-Nosjean MC. Characteristics of tertiary lymphoid structures in primary cancers. Oncoimmunology. 2013;2(12):e26836.
55. Nielsen JS, Nelson BH. Tumor-infiltrating B cells and T cells: Working together to promote patient survival. Oncoimmunology. 2012;1(9):1623-5.
56. Chang WC, Li CH, Huang SC, Chang DY, Chou LY, Sheu BC. Clinical significance of regulatory T cells and CD8+ effector populations in patients with human endometrial carcinoma. Cancer. 2010;116(24):5777-88.
57. Silverberg SG, Sasano N, Yajima A. Endometrial carcinoma in Miyagi Prefecture, Japan: histopathologic analysis of a cancer registry-based series and comparison with cases in American women. Cancer. 1982;49(7):1504-10.
58. Kondratiev S, Sabo E, Yakirevich E, Lavie O, Resnick MB. Intratumoral CD8+ T lymphocytes as a prognostic factor of survival in endometrial carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2004;10(13):4450-6.
59. Ambros RA, Kurman RJ. Combined assessment of vascular and myometrial invasion as a model to predict prognosis in stage I endometrioid adenocarcinoma of the uterine corpus. Cancer. 1992;69(6):1424-31.
60. Deligdisch L. Morphologic correlates of host response in endometrial carcinoma. American journal of reproductive immunology. 1982;2(1):54-7.
61. de Jong RA, Leffers N, Boezen HM, ten Hoor KA, van der Zee AG, Hollema H, et al. Presence of tumor-infiltrating lymphocytes is an independent prognostic factor in type I and II endometrial cancer. Gynecologic oncology. 2009;114(1):105-10.
62. Fattorossi A, Battaglia A, Ferrandina G, Buzzonetti A, Legge F, Salutari V, et al. Lymphocyte composition of tumor draining lymph nodes from cervical and endometrial cancer patients. Gynecologic oncology. 2004;92(1):106-15.
63. Yamamoto K, Masuko K, Takahashi S, Ikeda Y, Kato T, Mizushima Y, et al. Accumulation of distinct T cell clonotypes in human solid tumors. Journal of immunology. 1995;154(4):1804-9.
64. Contassot E, Preynat-Seauve O, French L, Huard B. Lymph node tumor metastases: more susceptible than primary tumors to CD8(+) T-cell immune destruction. Trends in immunology. 2009;30(12):569-73.
65. Garzetti GG, Ciavattini A, Goteri G, Romanini C. Nodal immune reactivity in FIGO (International Federation of Gyn-Ob) Stage I endometrial carcinoma: relationship with myometrial invasion. Acta obstetricia et gynecologica Scandinavica. 1995;74(9):723-8.
66. Arruvito L, Sanz M, Banham AH, Fainboim L. Expansion of CD4+CD25+and FOXP3+ regulatory T cells during the follicular phase of the menstrual cycle: implications for human reproduction. Journal of immunology. 2007;178(4):2572-8.

67. El-Hamarneh T, Hey-Cunningham AJ, Berbic M, Al-Jefout M, Fraser IS, Black K. Cellular immune environment in endometrial polyps. Fertility and sterility. 2013;100(5):1364-72.
68. Giatromanolaki A, Bates GJ, Koukourakis MI, Sivridis E, Gatter KC, Harris AL, et al. The presence of tumor-infiltrating FOXP3+ lymphocytes correlates with intratumoral angiogenesis in endometrial cancer. Gynecologic oncology. 2008;110(2):216-21.
69. Yamagami W, Susumu N, Tanaka H, Hirasawa A, Banno K, Suzuki N, et al. Immunofluorescence-detected infiltration of CD4+FOXP3+ regulatory T cells is relevant to the prognosis of patients with endometrial cancer. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society. 2011;21(9):1628-34.
70. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. The New England journal of medicine. 2003;348(3):203-13.
71. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. The New England journal of medicine. 2012;366(26):2455-65.
72. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. The New England journal of medicine. 2012;366(26):2443-54.
73. Iclozan C, Antonia S, Chiappori A, Chen DT, Gabrilovich D. Therapeutic regulation of myeloid-derived suppressor cells and immune response to cancer vaccine in patients with extensive stage small cell lung cancer. Cancer immunology, immunotherapy : CII. 2013;62(5):909-18.

Part III Validation of tumor-associated antigens

Part III

Chapter 3 Preclinical evaluation of survivin as target tumor-associated antigen for immunotherapy in uterine cancer

---

Vanderstraeten A, Tuyaerts S, Everaert T, Van Bree R, Verbist G, Luyten C and Amant F
Submitted

Preclinical evaluation of survivin as target tumor-associated antigen for immunotherapy in uterine cancer

ABSTRACT

Survivin is an anti-apoptotic protein, not expressed in terminally differentiated adult tissues, yet overexpressed in several tumors. Therefore, it is an interesting target for immunotherapeutic strategies. In addition to specific overexpression in tumors, tumor survival is mediated by survivin and hence tumor survival can be tackled by targeting survivin. Survivin expression in uterine cancer was validated by qRT-PCR and IHC. In addition, we evaluated survivin immunogenicity by analyzing spontaneous B cell and T cell responses in patients.

Survivin as a protein was expressed in only a minority of normal tissues, while being expressed in all of the currently analyzed uterine cancers, both endometrial carcinoma (n= 52) and uterine sarcoma (n=52). Survivin RNA transcripts were overexpressed in more aggressive tumors and survivin protein was overexpressed in recurrent endometrial tumors compared to primary tumors. These results further indicate the role of survivin in tumor cell survival.

Spontaneous T cell responses were seen in 5/24 endometrial cancer patients and 1/10 uterine sarcoma patients. No T cell responses were found in normal controls (n=17). Although increased antibody titers were found in more aggressive and far-advanced tumors, no differences in B cell responses were seen. Overall, when compared to normal controls, a B cell response was only measured in 1/35 uterine sarcoma patients.

In conclusion, we currently validated the presence of survivin in uterine cancer. In addition, spontaneous T cell responses were found in 17.6% of the total patient population. These data indicate that a survivin-specific tumor response may be induced spontaneously in patients, further fortifying the eligibility of survivin as an immunotherapeutic target.

INTRODUCTION

Several tumor-associated antigens have been described in cancer, one of which is survivin, a member of the inhibitor of apoptosis protein family. The exact function of survivin in the cell cycle and its role in the prevention of apoptosis in not yet fully understood, but it has become clear that survivin is involved in the control of several stages of the cell cycle [1]. Survivin is absent in fully developed adult tissues, except in proliferating cells. It has been shown to be expressed, for example, by thymocytes and bone marrow-derived stem cells [2]. In cancer however, survivin is up-regulated, among which in endometrial cancer and was associated with advanced disease and increased aggressiveness of tumors [3, 4]. Survivin expression has been linked to resistance to apoptosis, the formation of metastasis, bypass of cell cycle checkpoints and resistance to therapy [5]. Its presence has been shown to be a prognostic factor in several types of cancer and to have predictive value with regard to disease recurrence, summarized by Rödel et al. [6]. The location of survivin within cancer cells is of importance for its function, although data are inconsistent across several tumor types. Knauer et al. described that cytoplasmically located survivin is essential for its anti-apoptotic function [7]. These results were corroborated in colorectal cancer, where cytoplasmic survivin was associated with poor prognosis [8]. In contrast, nuclear localization of survivin has been associated with poor prognosis in lung cancer [9] and Merkel cell carcinoma [10], while it was inversely correlated with the occurrence of lymph node metastasis in esophageal cancer [11]. Additionally, a combination of

nuclear as well as cytoplasmic staining is a negative prognostic marker in high-grade astrocytomas [12].

This selective up-regulation in tumor cells, while being absent in normal tissue, in conjunction with its multiple roles in cancer, makes survivin a qualified antigenic target for cancer therapy [13]. Cancer immunotherapy targeting survivin has focused on different approaches in several cancer types, briefly reviewed by Altieri et al [5] and Andersen et al. [14].

In many types of cancer, spontaneous immune responses have been shown towards auto-antigens. A possible reason for the appearance of these responses is the presence of neo-antigens in tumors due to modifications of auto-antigens in tumors, differences in cellular localization, causing intracytoplasmic antigens to appear at the cellular surface as well as the release of cryptic epitopes and microvesicles from tumor cells [15]. This results in an increase of the immunogenicity of auto-antigens. These responses can be found both with regard to reactive T cells as well as auto-antibody responses. Auto-antibodies against several types of antigens, such as cancer testis antigens, mutation antigens, differentiation antigens and embryonal proteins have been shown in several types of tumors, reviewed by Reuschenbach, with varying outcomes concerning the presence of auto-antibodies and tumor stage as well as any correlation with prognosis [15, 16]. The most widely studied auto-antigen is p53, due to a high percentage of tumors bearing p53 mutations [17]. Quite some data are available on the presence of auto-antibodies for colorectal carcinoma and lung cancer [18-22].

Immune responses to survivin have been described in several types of tumors. Survivin-specific T cells were reported in lung tumors, multiple myeloma, breast cancer, leukemia and melanoma [14, 23, 24]. Auto-antibody responses were shown among others in colorectal cancer, lung cancer, gastrointestinal tumors, brain tumors and ovarian cancer [20, 22, 25-28].

In the current research we aim to validate the presence of survivin in uterine cancer and to evaluate the induction of spontaneous anti-survivin B and T cell responses. We hereby aim to gain insight into the possible eligibility of survivin for future use in immunotherapeutic strategies for uterine cancer patients.

MATERIALS AND METHODS

Patient samples and reagents

Biopsy material (tumor or normal endometrium) from uterine cancer patients and healthy controls was collected from the tissue biobank of the gynecologic oncology department at the University Hospital Leuven, either as snap-frozen biopsies for qRT-PCR or as paraffin-embedded tissue for immunohistochemistry (IHC).

For healthy donors, buffy coats were obtained from the Belgian Red Cross in ACD bags.

A panel of RNA isolated from normal tissues was purchased from Ambion (Life Technologies, Ghent, Belgium).

Peptide pools consisting of 15-mer peptides with an 11 aa overlap were obtained from Miltenyi Biotec (Leiden, the Netherlands) (survivin, CMVpp65, EBV BZLF1 and Influenza MP1).

Serum/plasma and PBMC isolation

Venous peripheral blood was collected in uncoated tubes or SST tubes (Vacutainer®, BD Bioscience, Erembodegem, Belgium) for serum samples and in EDTA-coated tubes for plasma samples (N = 8) and PBMC. Serum or plasma was separated from whole blood by centrifugation (10', 3000 rpm, 4°C). The resulting supernatant was aliquoted and stored at -80°C until use.
For healthy donor PBMC, buffy coats were diluted 1/3 in PBS and PBMC were isolated using Lymphoprep™ (AXIS-SHIELD, Dundee, Scotland) density gradient centrifugation and counted with Türck's solution. For patient samples, peripheral blood was diluted 1/2 in PBS and PBMC were isolated by the same procedure as above. PBMC were cryopreserved in 90% human AB serum (Sera Laboratories International, West Sussex, UK) containing 10% DMSO at 5-10 x 10^6 cells per vial using CoolCell freezing containers (BioCision, Larkspur, California, USA), and stored in liquid nitrogen until further use.

Quantitative real-time PCR

Total RNA was isolated from snap-frozen material using Trizol (Life Technologies, Ghent, Belgium), according to manufacturer's instructions. 0.2 µg RNA was reverse-transcribed in a total volume of 20 µl in a mixture containing 10X RT buffer, 5.5 mM $MgCl_2$, 10 mM dNTP's, 2.4 µM random hexamers, 0.5 U/µl RNase inhibitor and 50 U/µl Multiscribe reverse transcriptase (all from Life Technologies). The reaction was performed using ABI prism® 7000 Sequence Detection System (Life Technologies) as follows: 10 minutes at 25°C, 30 minutes at 48°C and 5 minutes at 95°C.

Quantitative real-time PCR (qRT-PCR) was performed by using ABI prism® 7000 Sequence Detection System (Life Technologies, Ghent, Belgium) and the data were analyzed with 7000 system software. All samples were analyzed in triplicate and testis cDNA was used as positive control. Survivin transcripts were detected using the human BIRC5 Taqman gene expression assay (Life Technologies, Ghent, Belgium). The following cycling program was used: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. Samples with housekeeping gene (β-actin and β-glucuronidase) cycle threshold (Ct) values above 30 were considered non-evaluable and omitted from analysis. Survivin expression levels were normalized to the geometric mean of β-actin and β-glucuronidase and the mean normalized expression (MNE) was calculated with Q-Gene software. Samples in which no Ct value could be measured were considered undetectable (i.e. no expression). Data are expressed as fold expression compared to the mean MNE value of normal endometrial samples. An increase of > 2-fold was considered as overexpression.

Immunohistochemistry

4.0 µm formol-fixed, paraffin-embedded tissue slides were deparaffinized and rehydrated using toluene and ethanol. Blocking of endogenous peroxidase was done for 30' using 0.5 % H_2O_2 in methanol. Heat-induced epitope retrieval (HIER) was performed for 2 h at 90°C in 0.01M Tris-HCl (pH 9.0) containing 1mM EDTA, after which aspecific antibody binding was blocked using a mixture of 2 % BSA and 1% non-fat milk powder in TBS. Primary antibody (Rb anti human survivin, 1.5 µg/ml (R&D Systems, Minneapolis, Minesota, USA)) was incubated overnight at 4°C. Visualization was performed using the EnVision+ system HRP-labelled polymers (DAKO, Leuven, Belgium) followed by DAB (Sigma-Aldrich, Diegem, Belgium) precipitation and hematoxylin counterstaining. All steps,

except the rehydration and blocking step, were followed by the appropriate washing step. The validation on normal tissues was done using a tissue microarray (TMA) (MTU951; Pantomics, Inc., Richmond, California, USA), following the same protocol.

Slide scoring

For evaluation of antigen expression, a scoring system was developed based on 2 different parameters; percentage of tumor cells staining positive for the antigen and the staining intensity. For each parameter, 3 different levels were determined. The percentage of tumor cells staining positive was divided in 1-25%, 25-50% and > 50%. The staining intensity was evaluated as weak (score 1), moderate (score 2) and strong (score 3). Based on these parameters a total score was assigned to each slide (Table 1). All slides were analyzed for the presence of tumor using a conventionally stained HE slide.

Table 1: scoring system for evaluation of TAA expression by IHC.

% positive tumor cells	Staining intensity	Assigned score
1-25	1	0
1-25	2	0
1-25	3	0
25-50	1	0
25-50	2	1+
25-50	3	2+
>50	1	0
>50	2	3+
>50	3	4+

Survivin antibody ELISA

Survivin auto-antibodies were analyzed in duplicate and determined using the commercially available Human anti-survivin (Surv) antibody ELISA Kit (CUSABIO, Wuhan, China), according to manufacturer's instructions. In brief, the provided recombinant calibrator was diluted to obtain a calibration curve ranging from 40 ng/ml – 0.625 ng/ml. Calibration curve and samples, diluted when appropriate, were incubated in the provided plate for 2 hours at 37°C. After incubation, antibody detection was done using a biotin-conjugated secondary antibody coupled to HRP-avidin, both of which incubated for 1 hour at 37°C. After incubation ranging from 10 to 30' with TMB substrate at 37°C, reaction was stopped and OD was measured at 450 nm, followed by subtraction of the OD of the blank sample or by subtracting the OD at a reference wavelength of 540 nm, following company protocol optimization. The two methods were compared and did not lead to significant differences in results.
For titer analysis, absolute values as calculated from the standard curve are used. Antibody responses are defined as the mean titers of the normal controls + 2 SD.

Detection of TAA-specific T cells

The CD137 assay was carried out in T cell medium (IMDM supplemented with 5% human AB serum, penicillin-streptomycin (Life Technologies, Ghent, Belgium) and L-glutamin (Life Technologies, Ghent, Belgium)) and the serum lot was pretested for assay performance. On day 1, 1-2 vials of PBMC were thawed in a 37°C water bath until only small ice crystals were visible. The cell suspension was

transferred to ice-cold RPMI1640 and centrifuged at 1500 rpm for 5 minutes at 4°C. The PBMC pellet was re-suspended in T cell medium, containing 10 U/ml DNase1 and left to recover at RT for 1 h. After 1 h, cells were counted with trypan blue and re-suspended in T cell medium at a concentration of 10^7 cells/ml. Cells were plated at 20-100 µl per well in half-well 96 well plates (Greiner Bio-One, Wemmel, Belgium) and cultured in the absence or presence of the indicated overlapping peptide pools at 0.6 nmol/ml for 16-20 h at 37°C / 5% CO2. After the incubation period, the cells were used for flow cytometric analysis.

Flow cytometry staining and analysis

Plates were centrifuged during 5 min at 1800 rpm and 4°C. After aspiration of the supernatant, cells were first washed with cold PBS (Life Technologies, Ghent, Belgium), centrifuged again and subsequently incubated for 30 min with 150 µl per well of a fixable viability dye (Fixable Viability Dye eFluor 506, eBioscience, Vienna, Austria) diluted at 1 µl/ml in PBS for discrimination of live and dead cells. After incubation, plates were centrifuged and cells were washed with PBS. Next, Fc receptor blocking was performed by adding 25 µl per well of a 10% normal goat serum (Sigma-Aldrich, Diegem, Belgium) solution in PBS/0.5% BSA. To identify antigen-specific T cells, the cells were stained with the following antibodies: CD4-FITC (BD Pharmingen, San Jose, California, USA), CD137-PE (BD Pharmingen, San Jose, California, USA), CD3-PerCp-Cy5.5 (BioLegend, San Diego, California, USA), CD19-PE-Cy7 (BioLegend, San Diego, California, USA) and CD8-APC-H7 (BD Pharmingen, San Jose, California, USA). All antibodies were titrated to optimal dilution before use. Acquisition was performed with a FACSCanto II flow cytometer using BD FACSDIVA software and 2.5-5 x 10^5 cells were acquired per sample. Photo Multiplier Tube (PMT) voltages were set using CS&T beads and compensation was calculated using single-stained samples. Data analysis was done using FACSDIVA.

The following gating strategy was used: first, dead cells were gated out using the viability dye and cells were gated on the lymphocyte population based on FSC/SSC properties. Within the lymphocyte population, a gate was set around $CD3^+$ $CD19^-$ cells and subsequently, the $CD4^+$ and $CD8^+$ population was gated in the $CD3^+$ population with exclusion of double-positive T cells. Finally, CD137 expression within the $CD4^+$ or $CD8^+$ population was evaluated. Background expression of CD137 by the T cells was determined on T cells from cultures without peptides. Positive reactivity to an antigen was defined as a minimum 2-fold increase in CD137 positivity compared to the unstimulated control. The gating of a representative sample is shown in Fig. 1.

These studies were conducted in a laboratory that operates under exploratory research principles using investigative protocols and assays.

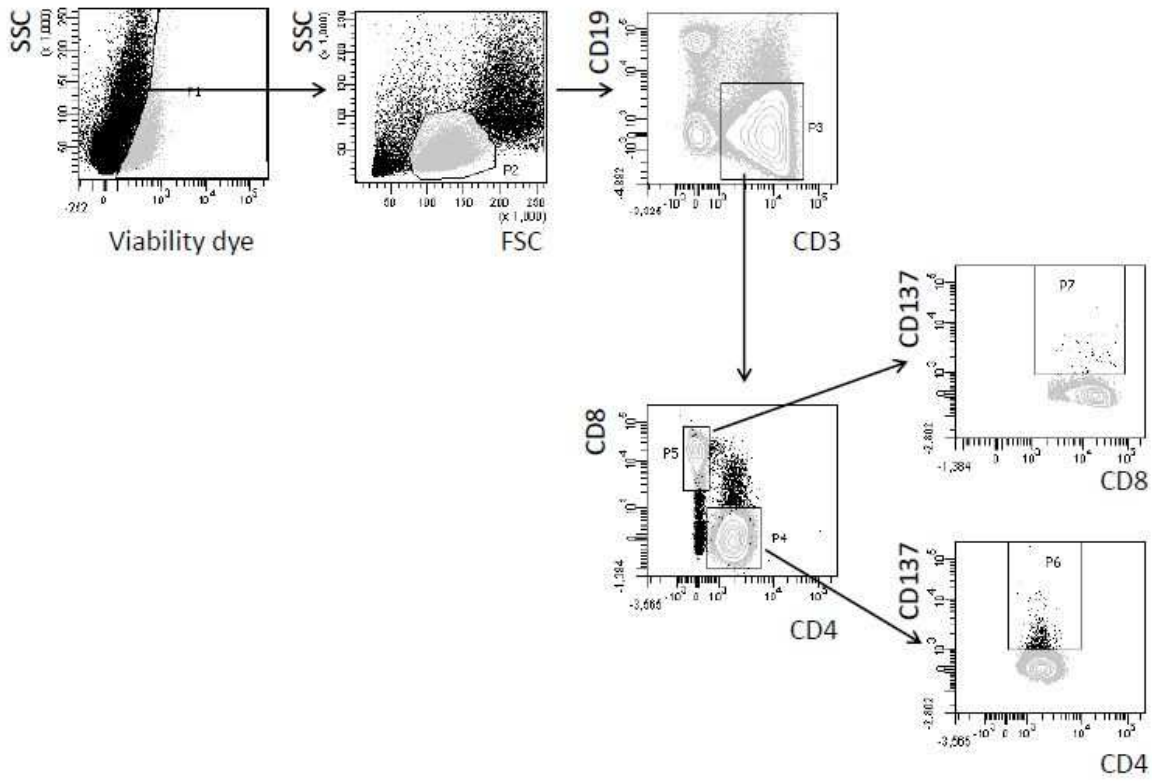


Figure 1: Gating strategy for detection of CD137^{+} T cells in a representative patient sample.

Statistics

Statistical analysis was performed using the GraphPad Prism 5.0 software. All sample groups were checked for Gaussian distribution using the D'Agostino and Pearson omnibus normality test followed by the appropriate (non)-parametric test when comparing 2 or more groups.

RESULTS

Survivin expression in normal tissues

Survivin expression levels were assessed in normal tissues and tumor samples. The presence of survivin was evaluated both at mRNA and at protein level.
Survivin mRNA expression was analyzed in a panel of normal tissues and showed the expected expression in thymus, testis and spleen. However, background levels of expression were also found in other tissues (Fig. 2A). Survivin was found to be expressed as a protein in only 25.4% of samples. Expression was found in the digestive tract (stomach, small intestine and colon); the male reproductive tract (testis, prostate) and in liver, kidney and the urinary bladder (Fig. 2B).

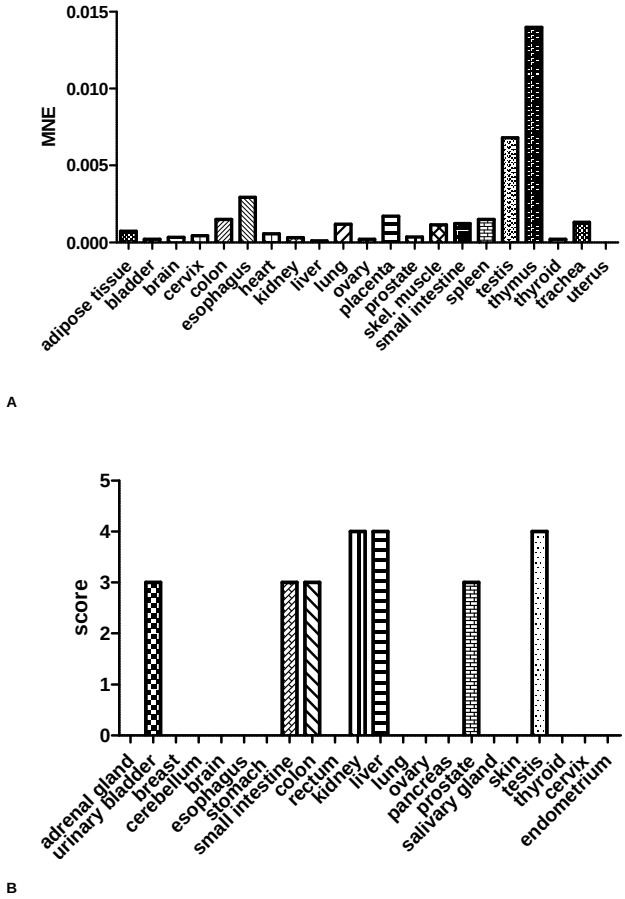


Figure 2: Survivin expression in normal tissues. A. Survivin mRNA expression, expressed as mean normalized expression. B. Survivin protein expression levels, expressed as evaluated expression score.

Survivin expression in uterine tumors

Analysis in normal endometrial samples and uterine tumors showed that survivin transcripts were detectable in 89 % of controls and in all EMCAR and US samples. Survivin mRNA was overexpressed in 71 % of EMCAR samples and 94 % of US samples compared to controls. Expression in US was significantly higher compared with EMCAR (p = 0.0108, Fig. 3A). With regard to clinicopathological tumor characteristics, survivin was found to be up-regulated in FIGO stage II versus FIGO stage I EMCAR (p < 0.05; Fig. 3B), as well as in grade 3 EMCAR versus grade 1 EMCAR (p < 0.01). Additionally, survivin was up-regulated in type II tumors, defined as grade 3 endometrioid and all serous and clear cell tumors, versus type I tumors, defined as grade 1 and 2 endometrioid tumors (p = 0.0025).

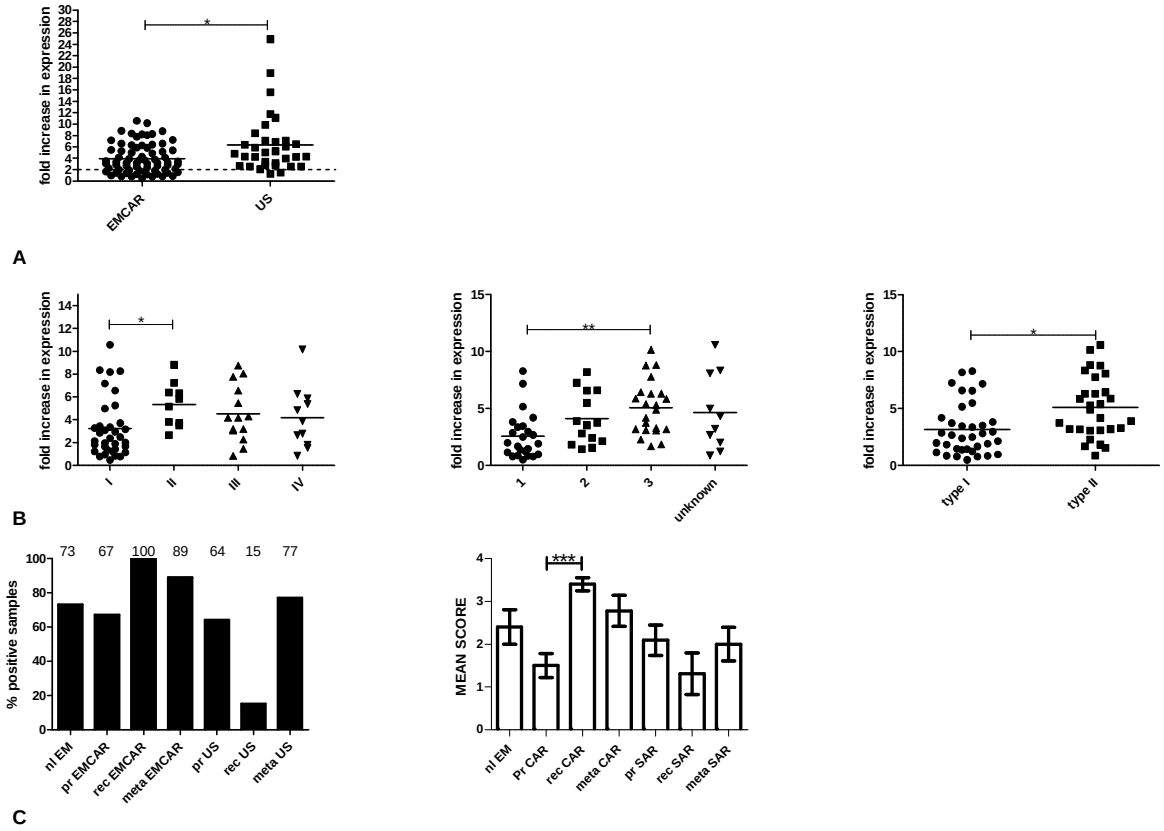


Figure 3: Survivin expression in uterine tumors. A. mRNA levels in EMCAR vs US (data expressed as fold increase compared to normal controls). B. left panel: survivin mRNA in EMCAR according to FIGO stage. Middle panel: survivin mRNA in EMCAR according to histological grade. Right panel: survivin mRNA in type I vs type II EMCAR. Data are expressed as fold increase compared to normal endometrium. C. left panel: percentage of samples positive for survivin protein. Numbers indicate the percentage of positive samples in each group. Right panel: survivin expresssion score in different tumor groups.
nl EM: normal endometrium, pr EMCAR: primary endometrial carcinoma, rec EMCAR: recurrent endometrial carcinoma, meta EMCAR: metastatic location endometrial carcinoma, pr US, primary uterine sarcoma, rec US: recurrent uterine sarcoma, meta US: metastatic location uterine sarcoma.
Normal distribution was verified in all groups before all other statistical analyses. Differences in expression between the tumor groups (A) was analyzed using the Mann Whitney test. Differences in expression in type I versus type II tumors was analyzed using an unpaired t test. All other groups were compared using the non-parametrical Kruskal-Wallis test followed by the Dunn's Multiple Comparison Test.

Table 2: clinical characteristics of IHC/qRT-PCR samples.

Tumor type	**IHC**		**qRT-PCR**	
Tumor type	EMCAR	US	EMCAR	US
Characteristics				
Total number of patients	48	47	70	34
Total number of tumors	52	52	70	34
Number of patients				
Primary	27	24	N/A	N/A
Recurrence	11	10	N/A	N/A
Metastasis	7	10	N/A	N/A
Primary + recurrence	0	1	N/A	N/A
Primary + metastasis	3	1	N/A	N/A
Metastasis+ recurrence	0	1	N/A	N/A
FIGO stage pr EMCAR[1] (number of tumors)				
I	10	N/A	35	N/A
II	4	N/A	10	N/A
III	7	N/A	14	N/A
IV	9	N/A	11	N/A
Histological grade EMCAR2 (number of tumors)				
1	7	N/A	24	N/A
2	3	N/A	14	N/A
3	32	N/A	22	N/A
Unknown	9	N/A	10	N/A
Histological subtype[3] (number of tumors)				
Endometrioid	17	N/A	48	N/A
Serous	21	N/A	11	N/A
Clear cell	9	N/A	3	N/A
Mixed	2	N/A	8	N/A
Carcinosarcoma[4]	2	18	N/A	11
Leiomyosarcoma	N/A	13	N/A	11
Rhabdomyosarcoma	N/A	7	N/A	2
Endometrial stromal sarcoma	N/A	10	N/A	7
Adenosarcoma	N/A	4	N/A	1
Mixed	N/A	-	N/A	1
unknown	N/A	-	N/A	1

[1] Only for IHC. Only primary tumors included; [2] Only for IHC. All tumors included; [3] Only for IHC. All tumors included, [4] Only for IHC. Only carinomatous tumor part included.

EMCAR samples expressed survivin protein in 85.3 % of pooled cases, with a significant up-regulation in recurrent tumors compared with the primary tumors ($p < 0.01$, Fig. 3C). In the US group, the pooled tumor population showed expression in only 52 % of the currently analyzed samples. For protein levels, no analysis was performed with regard to clinicopathological characteristics, due the small number of samples in each group. Clinical characteristics of all patient samples used for qRT-PCR analysis and IHC analysis are summarized in Table 2.

Both nuclear and cytoplasmic survivin expression was found in the majority of tumor samples. In the pooled population of all analyzed samples, combined expression was found in 38/52 EMCAR samples (73%) and in 33/52 (63%) US samples. On the contrary, 16/17 normal endometria (94%) showed only cytoplasmic expression.

Endogenous anti-survivin immunity

An important characteristic of an immunogenic TAA, is the ability to induce immune responses in patients. Therefore, we analyzed endogenous T and B cell responses against survivin in patients as well as healthy controls.

Survivin-specific T cells were assessed by culturing PBMC with overlapping peptides covering the whole antigen. Antigen-reactivity was assessed by analyzing CD137 up-regulation on both the $CD4^+$ and $CD8^+$ T cell subpopulations. Recall responses to viral antigens CMVpp65, EBV BZLF1 and Influenza Matrix Protein 1 were used as positive controls to assess immunocompetence. Reactivity with at least 1 of the 3 viral antigens was observed in 7/9 (78%) of benign controls, 21/24 (88%) of EMCAR patients and 8/10 (80%) of US patients (data not shown). In patients that did not mount a viral response, survivin-specific T cells could be measured in 14.2% of patients, while survivin-specific T cells were noted in 13.8% of patients with an anti-viral response (data not shown).

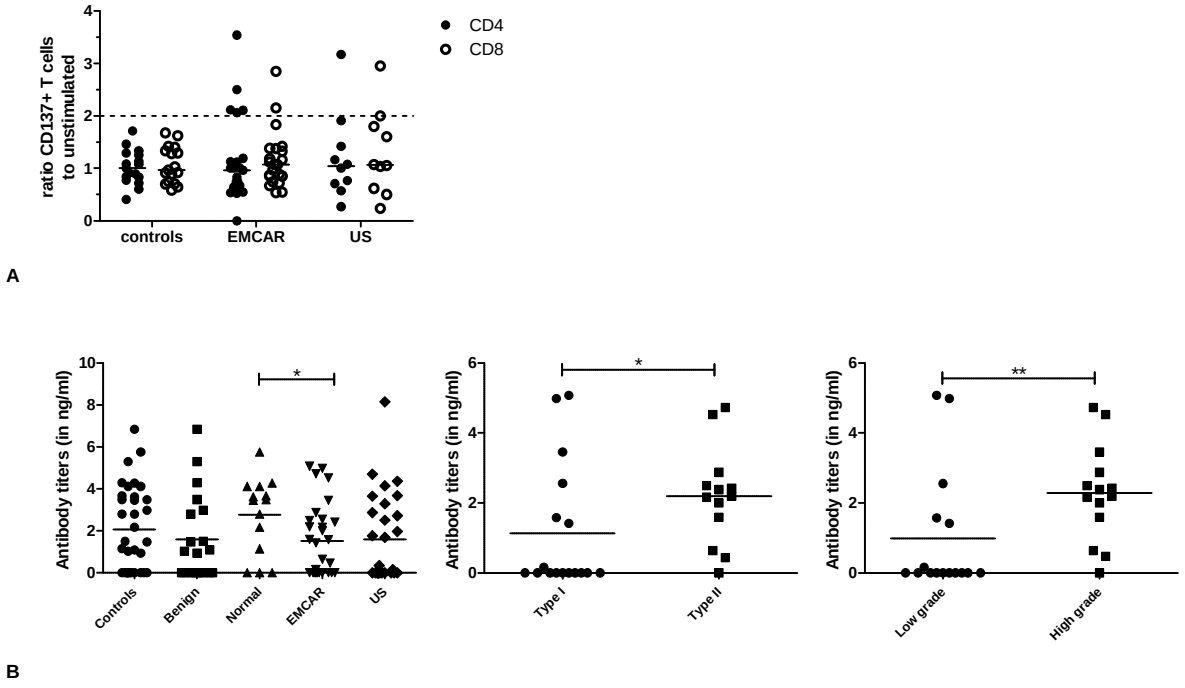


Figure 4: Spontaneous anti-survivin immune responses. A. $CD4^+$ and $CD8^+$ T cell responses in peripheral blood. Results are expressed as fold increase compared to unstimulated T cells. The minimal threshold of a 2-fold increase is indicated by the dashed line. B. B cell responses in peripheral blood. *Left panel:* comparison of titers at diagnosis. *Middle panel:* titers at diagnosis in type I versus type II endometrial tumors. *Right panel:* titers at diagnosis in low-grade versus high-grade endometrial tumors. Results are expressed as absolute titers. Antibody titers were analyzed separately compared to normal controls using the Mann Whitney test or an unpaired t test, depending on normal distribution. Type I versus type II tumors and low grade versus high grade tumors were analyzed using the Mann Whitney test.

Survivin-specific T cells could not be detected in control patients. However, survivin reactivity in EMCAR was noted in 5/24 patients for $CD4^+$ T cells and in 2/24 patients for $CD8^+$ T cells. In US, survivin responsiveness could be measured in 1/10 patients for $CD4^+$ T cells and in 1/10 patients for $CD8^+$ T cells (Fig. 4A). For some of the tested patients, we investigated whether levels of survivin-specific T cells correlate with survivin expression levels in the tumor, but we could not note a correlation (data not shown). Due to the limited number of available samples no analysis with regard to clinicopathological features could be performed (Table 3).

Table 3: Clinical characteristics of samples for spontaneous immune response.

	T cell response			**B cell response**		
Characteristics	**Controls**	**EMCAR**	**US**	**Controls**	**EMCAR**	**US**
Number of patients	17	24	10	34	39	35
Histology						
Benign	8	N/A	N/A	20	N/A	N/A
Normal	9	N/A	N/A	14	N/A	N/A
Endometrioid	N/A	16	N/A	N/A	24	N/A
Clear cell	N/A	1	N/A	N/A	2	N/A
Serous	N/A	4	N/A	N/A	10	N/A
Mixed	N/A	3	N/A	N/A	3	N/A
Carcinosarcoma	N/A	N/A	2	N/A	N/A	16
Leiomyosarcoma	N/A	N/A	3	N/A	N/A	10
Endometrial stromal sarcoma	N/A	N/A	2	N/A	N/A	6
Rhabdomyosarcoma	N/A	N/A	1	N/A	N/A	N/A
Adenosarcoma	N/A	N/A	N/A	N/A	N/A	2
Other	N/A	N/A	1	N/A	N/A	N/A
Unknown	N/A	N/A	1	N/A	N/A	1
Histological grade						
1	N/A	8	2	N/A	18	5
2	N/A	2	0	N/A	4	1
3	N/A	13	5	N/A	17	19
Unknown	N/A	1	3	N/A	N/A	10
Stage						
I	N/A	10	2	N/A	23	4
II	N/A	2	0	N/A	1	3
III	N/A	8	0	N/A	7	3
IV	N/A	3	6	N/A	8	8
recurrence	N/A	N/A		N/A	N/A	2
Unknown	N/A	1	2	N/A	N/A	15

B cell responses, defined by survivin-specific auto-antibodies were analyzed in plasma/serum. The total sample population of uterine tumor patients contains both samples that were taken prior to treatment and samples taken during treatment. In order to determine if there is an initial difference in titer at tumor diagnosis, all samples taken prior to surgery were analyzed separately. Unexpectedly, we found a significant decrease in antibody levels in EMCAR patients, compared to normal controls ($p = 0.0330$) as well as a trend towards decreased levels at diagnosis of US ($p = 0.0663$). No differences were found when comparing either tumor type compared to the total control group or the benign group separately (Fig. 4B).

Further analysis of the antibody titers in the patient groups, showed an increase in antibody titers at diagnosis in type II EMCAR, comprising serous and clear cell tumors as well as mixed and grade 3 endometrioid tumors compared to type I EMCAR, comprising grade 1 and grade 2 endometrioid tumors ($p = 0.0088$; Fig. 4B). In addition, we found a significant increase in titers in high grade tumors (grade 2 and 3) versus low grade tumors (grade 1; $p < 0,0102$; Fig. 4B). No difference in antibody titers was found when comparing locally advanced (FIGO I and II) and far-advanced tumors (FIGO III and IV) at diagnosis. In the US group, no differences were found between any of the histological types (data not shown). With the current data set of US patients, we were not able to perform in-depth analysis of histological grade nor FIGO stage, due to a fairly large number of unknown parameters (Table 3).

Although these significant differences in titers were seen, the vast majority of the measured titers did not reach the threshold for response, described in the material and methods section. A B cell response was measured in only 1/35 US patients and remarkably, in 1/8 patients with a benign gynecological affliction.

As described above for T cells, the correlation between antibody titers and the expression of survivin in the tumor was evaluated. For 9 EMCAR patients and 13 US patients tumor biopsies were stained and evaluated for survivin expression. In the EMCAR group 3/9 biopsies (33.3%) had a high score (total score 3 or 4) and 6/9 (66.7%) a low score (total score 0-2). In the US group 6/13 (46.1%) had a high score and 7/13 (53.9%) a low score. Next, we analyzed if the antibody titers in these patients correlated with the survivin expression level in the tumor. In the US group, samples from 2 patients were taken during chemotherapy following surgery. All other samples were taken preoperatively. No significant difference in the pooled EMCAR and US sample population was found concerning antibody titers when comparing the presence of tumors with a low survivin expression level compared to a high survivin level (data not shown).

DISCUSSION

We have currently validated the anti-apoptotic protein survivin as a possible target for immunotherapy in uterine cancer. To this end, both the presence of the antigen, as well as the immunogenicity were analyzed. Survivin was found to be present both in its RNA form and the translated protein. The currently obtained data in normal tissue were similar to previously published results, when comparing the same method of analysis, reviewed by Fukuda et al., except for the expression levels in skin and uterine cervix[29]. Several studies have already described the presence of survivin in EMCAR. Takai et al. showed that expression of survivin protein is correlated with histological grade, tumor stage and patient survival [30]. These results were, however, not corroborated in a later study by Pallares et al. [31]. In our study, survivin mRNA was found to be up-regulated in type II compared with type I EMCAR. Type II EMCAR comprises differentiation grade 3 endometrioid samples as well as serous and clear cell EMCAR, which are classified as high grade. The current RNA results thus corroborate the results of Takai et al [30]. We also found an up-regulation of survivin protein expression in recurrent carcinoma with respect to primary carcinoma, suggesting a survival advantage of the tumor cells leading to the development of the recurrent tumor.

The immunogenicity of an antigen, i.e. the ability to elicit an immune response is a key characteristic in order for the antigen to be eligible as a target for immunotherapy. Spontaneous T cell responses

against several TAA, mainly melanoma-associated antigens, have been described in several tumor types, both solid tumors and hematological tumors [32].

Spontaneous survivin-specific T cells have been described before in 9/23 multiple myeloma patients and 1/21 healthy donors [23]. In addition, Turksma et al. also described survivin-specific $CD8^+$ T cells in 3/5 patients with head and neck cancer [33].

To our knowledge, survivin-specific T cell have not been described in uterine cancer. We currently found specific T cell responses in 6/34 patients. Five EMCAR patients out of 24 and 1/10 US patients showed spontaneous survivin-specific T cells.

To date, little information is available concerning spontaneous auto-antibody levels, i.e. not induced by immunotherapy, in uterine tumors. In 2001, Bosscher et al. described the presence of anti-cathepsin D auto-antibodies in patients with endometrial cancer [34]. In 29/31 patients, antibodies against pro-cathepsin D were detected, whereas none of the controls showed responses [34]. To our knowledge, one other publication reports on survivin auto-antibodies in uterine tumors [21]. Among 11 different tumor types, ovarian and uterine tumors were included as gynecological malignancies. The authors report that from the total population that was analyzed, both gynecological tumor types and thyroid cancer showed very little immune response compared to other analyzed tumor types. Although not specified whether the uterine tumor population consisted of a combination of EMCAR and US samples or either, a survivin antibody response was found in 4/27 (14.8%) of analyzed uterine tumor samples, whereas in ovarian cancer only 1/39 (2.6%) tumor samples showed positivity [21]. In our sample group, out of a pooled tumor population of 74 tumor samples, only 1 sample was considered positive (1.3%). Our results corroborate the finding that survivin auto-antibody responses are low in uterine tumors. However, the responses detected by Megliorino et al. are still considerably higher than the current data [21]. We believe the difference can be explained by the threshold criteria that define an immune response. Like for many other publications on detection of auto-antibodies that use a threshold of the mean OD measured for the control group + 2 or 3 SD, the latter was also used by Megliorino et al [21]. In our analysis, however, a cut-off value based on the extrapolated titer + 2 SD rather than on the raw OD value was used. This method is likely more stringent, since standard deviations of extrapolated values may be larger. In addition, in the current sample population, actual titers compared to a standard curve with known concentrations of survivin antibodies were analyzed, instead of a comparison of OD values between different groups. In the current data set, with application of these threshold criteria, none of the normal control samples were above threshold. In one patient with a benign gynecological affliction, an auto-antibody response was measured, which may in fact be explained by the presence of the benign condition, which may elicit anti-survivin responses as well.

Data on a correlation between the presence of an antigen in the tumor and a corresponding immune response are not always consistent, as reviewed by Reuschenbach et al. [16]. A link between the presence of anti-survivin antibody responses and the expression of survivin mRNA was found in patients with head and neck tumors [35]. In our results, we did not find any correlation between increased titers, below response threshold, and the presence of low or high survivin expression levels. In addition, no correlation was found between the presence of survivin in the tumor and the presence of survivin-reactive T cells in peripheral blood.

When analyzing antibody titers according to histopathological characteristics, we showed that patients suffering from EMCAR with a more aggressive phenotype (type II EMCAR) or high-grade EMCAR showed increased anti-survivin antibody titers, indicating some reaction of the immune system although it does not reach the threshold of a definite immune response. This increase in

titers corroborates results found in head and neck tumors by Chang et al. [36] and Eto et al. [37], who showed increased responses in patients with far-advanced tumors and Li et al. and Taylor et al, who showed increased survivin auto-antibody levels in advanced ovarian cancer [25, 26].

In conclusion, we currently validated the presence of the anti-apoptotic protein survivin in uterine tumors. To our knowledge, this is the first paper to describe its presence in uterine sarcoma.

To further evaluate the validity of survivin as an immunotherapeutic target, we investigated the occurrence of spontaneous T and B cell responses directed against survivin. We found a B cell response in only 1 patient, while T cell responses could be detected in 6/34 patients.

These data collectively indicate that survivin poses as a valid candidate for future immunotherapeutic strategies in the treatment uterine cancer.

REFERENCES

1. Yang D, Welm A, Bishop JM. Cell division and cell survival in the absence of survivin. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(42):15100-5.
2. Altieri DC. Validating survivin as a cancer therapeutic target. Nature reviews Cancer. 2003;3(1):46-54.
3. Lambropoulou M, Papadopoulos N, Tripsianis G, Alexiadis G, Pagonopoulou O, Kiziridou A, et al. Co-expression of survivin, c-erbB2, and cyclooxygenase-2 (COX-2): prognostic value and survival of endometrial cancer patients. Journal of cancer research and clinical oncology. 2010;136(3):427-35.
4. Brunner A, Riss P, Heinze G, Brustmann H. pHH3 and survivin are co-expressed in high-risk endometrial cancer and are prognostic relevant. British journal of cancer. 2012;107(1):84-90.
5. Altieri DC. Targeting survivin in cancer. Cancer letters. 2013;332(2):225-8.
6. Rodel F, Sprenger T, Kaina B, Liersch T, Rodel C, Fulda S, et al. Survivin as a prognostic/predictive marker and molecular target in cancer therapy. Current medicinal chemistry. 2012;19(22):3679-88.
7. Knauer SK, Kramer OH, Knosel T, Engels K, Rodel F, Kovacs AF, et al. Nuclear export is essential for the tumor-promoting activity of survivin. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2007;21(1):207-16.
8. Qi G, Tuncel H, Aoki E, Tanaka S, Oka S, Kaneko I, et al. Intracellular localization of survivin determines biological behavior in colorectal cancer. Oncology reports. 2009;22(3):557-62.
9. Shinohara ET, Gonzalez A, Massion PP, Chen H, Li M, Freyer AS, et al. Nuclear survivin predicts recurrence and poor survival in patients with resected nonsmall cell lung carcinoma. Cancer. 2005;103(8):1685-92.
10. Kim J, McNiff JM. Nuclear expression of survivin portends a poor prognosis in Merkel cell carcinoma. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2008;21(6):764-9.
11. Hui MK, Lai KK, Chan KW, Luk JM, Lee NP, Chung Y, et al. Clinical correlation of nuclear survivin in esophageal squamous cell carcinoma. Medical oncology. 2012;29(5):3009-16.
12. Saito T, Arifin MT, Hama S, Kajiwara Y, Sugiyama K, Yamasaki F, et al. Survivin subcellular localization in high-grade astrocytomas: simultaneous expression in both nucleus and cytoplasm is negative prognostic marker. Journal of neuro-oncology. 2007;82(2):193-8.
13. Lladser A, Sanhueza C, Kiessling R, Quest AF. Is survivin the potential Achilles' heel of cancer? Advances in cancer research. 2011;111:1-37.
14. Andersen MH, Pedersen LO, Capeller B, Brocker EB, Becker JC, thor Straten P. Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in situ as well as ex vivo in cancer patients. Cancer research. 2001;61(16):5964-8.
15. Heo CK, Bahk YY, Cho EW. Tumor-associated autoantibodies as diagnostic and prognostic biomarkers. BMB reports. 2012;45(12):677-85.

16. Reuschenbach M, von Knebel Doeberitz M, Wentzensen N. A systematic review of humoral immune responses against tumor antigens. Cancer immunology, immunotherapy : CII. 2009;58(10):1535-44.
17. Suppiah A, Greenman J. Clinical utility of anti-p53 auto-antibody: systematic review and focus on colorectal cancer. World journal of gastroenterology : WJG. 2013;19(29):4651-70.
18. Chen H, Werner S, Tao S, Zornig I, Brenner H. Blood autoantibodies against tumor-associated antigens as biomarkers in early detection of colorectal cancer. Cancer letters. 2014.
19. Healey GF, Lam S, Boyle P, Hamilton-Fairley G, Peek LJ, Robertson JF. Signal stratification of autoantibody levels in serum samples and its application to the early detection of lung cancer. Journal of thoracic disease. 2013;5(5):618-25.
20. Karanikas V, Khalil S, Kerenidi T, Gourgoulianis KI, Germenis AE. Anti-survivin antibody responses in lung cancer. Cancer letters. 2009;282(2):159-66.
21. Megliorino R, Shi FD, Peng XX, Wang X, Chan EK, Tan EM, et al. Autoimmune response to anti-apoptotic protein survivin and its association with antibodies to p53 and c-myc in cancer detection. Cancer detection and prevention. 2005;29(3):241-8.
22. Rohayem J, Diestelkoetter P, Weigle B, Oehmichen A, Schmitz M, Mehlhorn J, et al. Antibody response to the tumor-associated inhibitor of apoptosis protein survivin in cancer patients. Cancer research. 2000;60(7):1815-7.
23. Grube M, Moritz S, Obermann EC, Rezvani K, Mackensen A, Andreesen R, et al. CD8+ T cells reactive to survivin antigen in patients with multiple myeloma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13(3):1053-60.
24. Karanikas V, Soukou F, Kalala F, Kerenidi T, Grammoustianou ES, Gourgoulianis KI, et al. Baseline levels of CD8+ T cells against survivin and survivin-2B in the blood of lung cancer patients and cancer-free individuals. Clinical immunology. 2008;129(2):230-40.
25. Li L, Wang K, Dai L, Wang P, Peng XX, Zhang JY. Detection of autoantibodies to multiple tumor-associated antigens in the immunodiagnosis of ovarian cancer. Molecular medicine reports. 2008;1(4):589-94.
26. Taylor DD, Gercel-Taylor C, Parker LP. Patient-derived tumor-reactive antibodies as diagnostic markers for ovarian cancer. Gynecologic oncology. 2009;115(1):112-20.
27. Soling A, Plugge EM, Schmitz M, Weigle B, Jacob R, Illert J, et al. Autoantibodies to the inhibitor of apoptosis protein survivin in patients with brain tumors. International journal of oncology. 2007;30(1):123-8.
28. Yagihashi A, Asanuma K, Nakamura M, Araya J, Mano Y, Torigoe T, et al. Detection of anti-survivin antibody in gastrointestinal cancer patients. Clinical chemistry. 2001;47(9):1729-31.
29. Fukuda S, Pelus LM. Survivin, a cancer target with an emerging role in normal adult tissues. Molecular cancer therapeutics. 2006;5(5):1087-98.
30. Takai N, Miyazaki T, Nishida M, Nasu K, Miyakawa I. Survivin expression correlates with clinical stage, histological grade, invasive behavior and survival rate in endometrial carcinoma. Cancer letters. 2002;184(1):105-16.
31. Pallares J, Martinez-Guitarte JL, Dolcet X, Llobet D, Rue M, Palacios J, et al. Survivin expression in endometrial carcinoma: a tissue microarray study with correlation with PTEN and STAT-3. International journal of gynecological pathology : official journal of the International Society of Gynecological Pathologists. 2005;24(3):247-53.
32. Nagorsen D, Scheibenbogen C, Marincola FM, Letsch A, Keilholz U. Natural T cell immunity against cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2003;9(12):4296-303.
33. Turksma AW, Bontkes HJ, Ruizendaal JJ, Scholten KB, Akershoek J, Rampersad S, et al. Exploring dendritic cell based vaccines targeting survivin for the treatment of head and neck cancer patients. Journal of translational medicine. 2013;11:152.
34. Bosscher JR, Gercel-Taylor C, Watkins CS, Taylor DD. Epitope recognition by anti-cathepsin D autoantibodies in endometrial cancer patients. Gynecologic oncology. 2001;81(2):138-43.

35. Uemura N, Kodama S, Nomi N, Okamoto T, Suzuki M. Correlation between anti-survivin antibody and survivin mRNA expression in head and neck cancer patients. Acta oto-laryngologica. 2010;130(8):959-65.
36. Chang JT, Wong FH, Liao CT, Chen IH, Wang HM, Cheng AJ. Enzyme immunoassay for serum autoantibody to survivin and its findings in head-and-neck cancer patients. Clinical chemistry. 2004;50(7):1261-4.
37. Eto M, Kodama S, Uemura N, Suzuki M. Antibody responses to survivin and their clinical significance in patients with head and neck cancer. Head & neck. 2007;29(12):1128-35.

Part III

Chapter 4 Validation of tumor antigens as immunotherapeutic targets in uterine cancer

Vanderstraeten A, Tuyaerts S, Everaert T, Van Bree R, Verbist G, Luyten C and Amant F
Unpublished data

Validation of tumor antigens as immunotherapeutic targets in uterine cancer

ABSTRACT

Immunotherapy for uterine cancers is still in its early days. Although quite some information is known about the presence of TAA in uterine cancer, its implementation into immunotherapeutic regimens has so far not been pursued to large extent. We currently validate TAA in uterine cancer patients in order to ascertain future applicability in dendritic cell based immunotherapy.
The presence of 5 different TAA; BORIS, MUC1, hTERT, MAGE-A3 and Sp17 was validated in human tumor samples of endometrial carcinoma as well as uterine sarcoma by IHC and/or qRT-PCR. In addition, we have analyzed the immunogenicity of these antigens by determining the spontaneous TAA-specific T cell responses in patient blood.
All the currently analyzed TAA were found to be present in the majority of both endometrial carcinoma and uterine sarcoma, with MUC1 being preferentially expressed in uterine sarcomas containing an epithelial component.
Spontaneous T cell responses against the analyzed TAA were detected in only a minority of patients. In addition, we found spontaneous T cell responses in patients with benign gynecological afflictions.
Taken together, the currently obtained data provide further indications of the immunogenicity of TAA in uterine cancer, fortifying their validity as targets in immunotherapeutic regimens.

INTRODUCTION

Cancer vaccination, based on the recognition of the so-called tumor-associated antigens (TAA), is a valid therapeutic option for many types of cancer. Numerous different TAA have already been described to date. In 2009, Cheever et al. ranked a list of known TAA according to a whole array of characteristics, such as tissue expression, presentation in MHC context, and immunogenicity [1]. Several TAA have already been described in various tumor types and tested in (pre)clinical immunotherapeutic settings with varying success [2]. Most data have been gathered in among others melanoma [3-6], glioblastoma [7, 8], renal cell carcinoma (RCC) [9], prostate cancer [8] and hematological malignancies [10].
Some of these known antigens have already been described in uterine cancer. However, even though TAA have been described in uterine tumors, only few studies were done to verify their potential use in immunotherapeutic strategies. Uterine tumors have been shown to be immunogenic which is exemplified by the presence of tumor-infiltrating lymphocytes and macrophages [11, 12]. Some immunotherapy studies have already been done in uterine tumors using WT1 as target antigen [5, 13, 14]. Other (pre)clinical studies in uterine tumors based on the targeting of antigens have mainly focused on the use of whole tumor lysate [15, 16]. The current research is focused on the validation of some of the described TAA in uterine tumors. In specific, we have analyzed the following antigens: brother of the regulator of imprinted sites (BORIS), mucin-1 (MUC1), human telomerase reverse transcriptase (hTERT), sperm protein 17 (Sp17) and melanoma-associated antigen A3 (MAGE-A3). BORIS, Sp17 and MAGE-A3 are members of the cancer-testis antigen family. In normal tissues, their expression is restricted to male germ cells, fetal ovary, placenta and immature gametes [17], while they are expressed in several types of cancer, among which breast cancer [18, 19], making them attractive targets for immunotherapeutic purposes. hTERT is considered a universal TAA. This antigen

plays a role in the survival of tumor cells. It is absent in adult tissues, with the exception of proliferating tissues, while being up-regulated in dividing tumor cells, which fortifies its suitability as target TAA [20, 21]. MUC1 is an epithelial antigen, constitutively expressed on normal epithelium in its wild-type form. The TAA MUC1, however, discriminates itself from the wild-type form by a hypoglycosylation pattern, exposing new epitopes, which can attract antigen-specific T cells [22]. These antigens were arbitrarily selected based on an analysis of the available data of these antigens concerning (a) their presence in uterine tumors, (b) their ranking in the prioritization list described above, (c) available data on their application in immunotherapeutic regimens in other tumor types, and (d) their specific expression/function in tumorigenesis.
We currently aim to validate the presence of these TAA in endometrial carcinomas and evaluate their expression in uterine sarcoma, which has, to our knowledge, not been described before. In addition, we evaluate their immunogenicity by analyzing the naturally-occurring *in vivo* TAA-specific T cell responses. The combined data will give a view on the applicability of the currently investigated TAA for immunotherapy in uterine cancer patients.

MATERIALS & METHODS

Patient samples, cell lines and reagents

Biopsy material from uterine cancer patients and healthy controls was collected from the tissue biobank of the gynecologic oncology department at the University Hospital Leuven. Whole blood was collected from uterine cancer patients or patients with benign gynecological diseases through venipuncture using EDTA-coated BD vacutainer tubes (BD Diagnostics, Erembodegem, Belgium); buffy coats of healthy controls, collected in ACD bags, were purchased from the Belgian Red Cross. Freshly collected blood was stored at room temperature and processed within 24 hours.
Informed consent was obtained from all patients donating blood and/or tumor tissue prior to inclusion in the study.
Expression levels in normal tissues were analyzed using RNA isolated from normal tissues, purchased from Ambion (Life Technologies, Ghent, Belgium) and/or a tissue microarray (TMA) (MTU951; Pantomics, Inc., Richmond, California, United States).
The commercial cell lines SKOV-3, OVCAR-3, RL-95-2, HEC-1 and SK-UT-1 were obtained from ATCC.
Peptide pools consisting of 15-mer peptides with an 11 aa overlap were obtained from Miltenyi Biotec (Leiden, the Netherlands) (MAGE-A3, CMVpp65, EBV BZLF1 and Influenza MP1), JPT Peptide Technologies GmbH (Berlin, Germany) (MUC1, VNTR repeat region) or Thermo Scientific (Beersel, Belgium) (Sp17).

RNA isolation and reverse transcription reaction

Total RNA was isolated from snap-frozen material using Trizol (Life Technologies, Ghent, Belgium), according to manufacturer's instructions. 0.2 µg RNA was reverse-transcribed in a total volume of 20 µl in a mixture containing 10X RT buffer, 5.5 mM $MgCl_2$, 10 mM dNTP's, 2.4 µM random hexamers, 0.5 U/µl RNase inhibitor and 50 U/µl Multiscribe reverse transcriptase (all from Life Technologies). The reaction was performed using ABI prism® 7000 Sequence Detection System (Life Technologies) as follows: 10 minutes at 25°C, 30 minutes at 48°C and 5 minutes at 95°C.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed by using ABI prism® 7000 Sequence Detection System (Life Technologies) and the data were analyzed with 7000 system software. Tumor cDNA was analyzed together with testis cDNA as positive control. qRT-PCR reactions were performed in triplicate for all samples. Thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. Primers used for the different genes are indicated in Table 1. TAA gene expression levels were normalized to the geometric mean of β-actin and β-glucuronidase and the mean normalized expression (MNE) was calculated with Q-Gene software. Samples in which cycle threshold (Ct) values of housekeeping genes (β-actin and β-glucuronidase) were above 30 were considered to be of insufficient quality and omitted from analysis. Samples in which no Ct value for a gene of interest could be measured, were considered undetectable (i.e. no expression). All PCR data were expressed as fold change compared to the mean MNE of normal endometrium samples. TAA were considered to be overexpressed in case of ≥ two-fold increase. Statistical analysis to calculate differences in expression levels between groups were performed with GraphPad Prism software, using the appropriate (non)-parametric test following initial analyses to determine normal distribution of results using the D'Agostino and Pearson omnibus normality test.

Table 1: Primers used for qRT-PCR

TAA	**Primers and probe**
BORIS	Human CTCFL Taqman gene expression assay from Applied Biosystems (Life Technologies)
MAGE-A3	Sense primer, 5'-GTCGTCGGAAATTGGCAGTAT-3' Antisense primer, 5'-GCAGGTGGCAAAGATGTACAA-3' Probe, 5'-6FAM-AAAGCTTCCAGTTCCTT-MGB-3'
Sp17	Human SPA17 Taqman gene expression assay from Applied Biosystems (Life Technologies)
hTERT	Human TERT Taqman gene expression assay from Applied Biosystems (Life Technologies)
β-actin	Human ACTB (beta actin) Endogenous Control from Applied Biosystems (Life Technologies)
β-glucuronidase	Human GUSB (beta glucuronidase) Endogenous Control from Applied Biosystems (Life Technologies)

Immunohistochemistry

For all stainings, 4.0 µm phormol-fixed, paraffin-embedded tissue sections were stained. Sections were first deparaffinized and rehydrated in ethanol. Next, endogenous peroxidase was blocked in 0.5% (MUC1) or 3% H_2O_2 (hTERT) in methanol for 30 minutes. For hTERT, heat-induced epitope retrieval (HIER) was performed at 85°C. For MUC1, the same step was performed for 1h at 90°C in citrate (pH 6.0), after which the slides were cooled down and primary antibody incubation was performed. Ms α Hu MUC1 (DAKO (Leuven, Belgium; 1.15 µg/ml) incubated for 2 h at room temperature (RT). Rb anti Hu TERT (Rockland (Gilbertsville, Pensylvania, United States; 2.5 µg/ml) incubated overnight at 4°C. For all antigens, EnVision+ system HRP-labelled polymers (DAKO) were used as secondary antibodies and incubated for 30 minutes at RT. Visualization was performed using 3,3'-Diaminobenzidine (DAB) and hematoxylin counterstaining.

The validation of the tumor associated antigens on normal tissues was done using a tissue microarray (TMA) (MTU951; Pantomics, Inc. Richmond, California, United States), following the same protocols.

Slide scoring

For evaluation of antigen expression, a scoring system was developed based on 2 different parameters; percentage of tumor cells staining positive for the antigen and the staining intensity. For each parameter, 3 different levels were determined. The percentage of tumor cells staining positive was divided in 1-25%, 25-50% and > 50%. The staining intensity was evaluated as weak (score 1), moderate (score 2) and strong (score 3). Based on these parameters a total score was assigned to each slide (Table 2). All slides were analyzed for the presence of tumor using a conventionally stained HE slide.

Table 2: scoring system for evaluation of TAA expression by IHC

% positive tumor cells	Staining intensity	Assigned score
1-25	1	0
1-25	2	0
1-25	3	0
25-50	1	0
25-50	2	1+
25-50	3	2+
>50	1	0
>50	2	3+
>50	3	4+

PBMC isolation

For healthy donors, buffy coats were diluted 1/3 in PBS and PBMC were isolated using Lymphoprep™ (AXIS-SHIELD, Dundee, Scotland) density gradient centrifugation according to manufacturer's instructions and counted with Türck's solution. For patient samples, peripheral blood was diluted 1/2 in PBS and isolated following the same protocol. The median PBMC number obtained was $8.5x10^6$ cells per 10 ml of whole patient blood. PBMC were cryopreserved in 90% human AB serum (Sera Laboratories International, West Sussex, United Kingdom) containing 10% DMSO at 5-10 x 10^6 cells per vial using CoolCell freezing containers (BioCision, Larkspur, California, United States). The freezing container was placed at -80°C and vials were transported to liquid nitrogen after 16-48 h and stored until further use.

Detection of TAA-specific T cells

The CD137 assay was carried out in T cell medium (IMDM supplemented with 5% human AB serum, penicillin-streptomycin (Life Technologies) and L-glutamin (Life Technologies)) and the serum lot was pretested for assay performance. On day 1, 1-2 vials of PBMC were thawed in a 37°C water bath until only small ice crystals were visible. The cell suspension was transferred to ice cold RPMI1640 and centrifuged at 1500 rpm for 5 minutes at 4°C. The PBMC pellet was re-suspended in T cell medium, containing 10 U/ml DNase1 and left to recover at RT for 1 h. After 1 h, cells were counted with trypan blue and re-suspended in T cell medium at a concentration of 10^7 cells/ml. Cells were plated at 20-100 µl per well in half-well 96 well plates (Greiner Bio-One, Wemmel, Belgium) and cultured in

the absence or presence of the indicated overlapping peptide pools at 0.6 nmol/ml for 16-20 h at 37°C / 5% CO_2. After the incubation period, the cells were used for flow cytometric analysis.

Flow cytometry staining and analysis

Plates were centrifuged during 5 min at 1800 rpm and 4°C. After aspiration of the supernatant, cells were first washed with cold PBS (Life Technologies, Ghent, Belgium), centrifuged again and subsequently incubated for 30 min with 150 µl per well of a fixable viability dye (Fixable Viability Dye eFluor 506, eBioscience, Vienna, Austria) diluted at 1 µl/ml in PBS for discrimination of live and dead cells. After incubation, plates were centrifuged and cells were washed with PBS. Next, Fc receptor blocking was performed by adding 25 µl per well of a 10% normal goat serum (Sigma-Aldrich) solution in PBS/0.5% BSA. To identify antigen-specific T cells, the cells were stained with the following antibodies: CD4-FITC (BD Pharmingen, San Jose, California, United States), CD137-PE (BD Pharmingen, San Jose, California, United States), CD3-PerCp-Cy5.5 (BioLegend, San Diego, California, United States), CD19-PE-CY7 (Biolegend, San Diego, California, United States) and CD8-APC-H7 (BD Pharmingen, San Jose, California, United States). All antibodies were titrated to optimal dilution before use. Acquisition was performed with a FACSCanto II flow cytometer using BD FACSDIVA software v6.1.3 and 2.5-5 x 10^5 cells were acquired per sample. Photo Multiplier Tube (PMT) voltages were set using CS&T beads and compensation was calculated using single-stained samples. Data analysis was done using FACSDIVA v6.1.3.
The following gating strategy was used: first, dead cells were gated out using the viability dye and cells were gated on the lymphocyte population based on FSC/SSC properties. Within the lymphocyte population, a gate was set around $CD3^+$ $CD19^-$ cells and subsequently, the $CD4^+$ and $CD8^+$ population was gated in the $CD3^+$ population with exclusion of double-positive T cells. Finally, CD137 expression within the $CD4^+$ or $CD8^+$ population was evaluated. Background expression of CD137 by the T cells was determined on T cells from cultures without peptides. Positive reactivity to an antigen was predefined as a minimum 2-fold increase in CD137 positivity compared to the unstimulated control. The gating of a representative sample is shown in Fig. 1.
These studies were conducted in a laboratory that operates under exploratory research principles using investigative protocols and assays.

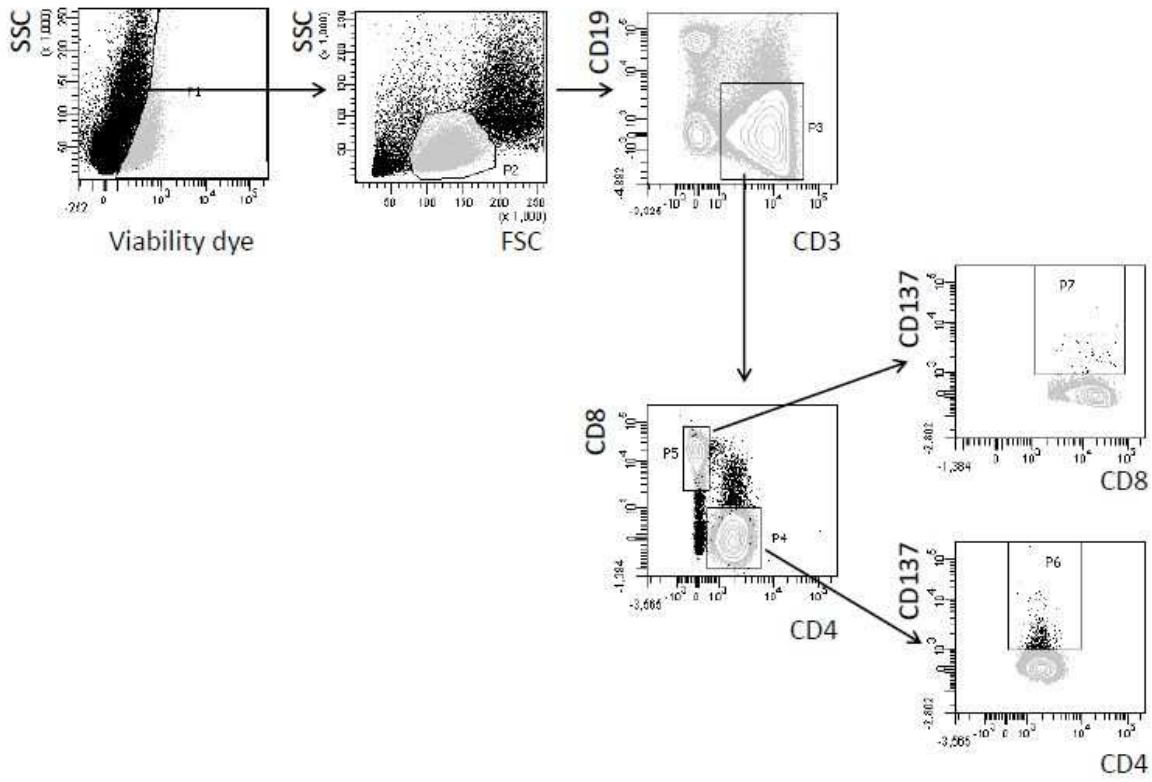


Figure 1: Gating strategy for detection of CD137^{+} T cells in a representative patient sample.

RESULTS

TAA expression in normal human tissues and tumor cell lines at mRNA level

Prior to testing primary patient material, mRNA expression levels of the different TAA were assessed on a panel of normal human tissues. For the currently selected antigens, PCR analysis was performed for all antigens, except for MUC1. The difference between the wild-type and tumor antigen MUC1 is a difference in glycosylation pattern. This is a post-translational modification and can thus not be detected at the level of RNA. As expected, the cancer-testis antigens BORIS, MAGE-A3 and Sp17 were expressed at the highest levels in testis, but for BORIS and Sp17 a fairly high level of background expression could also be observed in other tissues. MAGE-A3 expression was more restricted to testis (Fig. 2A). With regard to the universal TAA hTERT, the highest expression levels were found, as expected, in tissues with a lot of proliferative cells, such as spleen, testis, and thymus (Fig. 2A).
TAA expression was also assessed on a small selection of commercially available gynecological tumor cell lines. All TAA were expressed at medium to high levels in these cell lines, except for MAGE-A3 which showed lower expression levels (Fig. 2B).

	Cancer-testis antigens							
	BORIS		MAGE-A3		Sp17		hTERT	
	MNE	Level	MNE	Level	MNE	Level	MNE	Level
ADIPOSE	1,86E-04	++	8,971E-07	-	1,48E-03	+++	5,65E-06	+/-
BLADDER	4,10E-04	++	1,832E-06	+/-	4,50E-03	+++	6,05E-06	+/-
BRAIN	5,44E-04	++	9,749E-07	-	3,55E-02	++++	4,02E-05	+
CERVIX	1,34E-04	++	1,032E-06	+/-	7,05E-03	+++	4,29E-06	+/-
COLON	5,50E-03	+++	1,52E-06	+/-	3,44E-03	+++	1,05E-04	++
ESOPHAGUS	6,22E-04	++	1,255E-06	+/-	7,77E-03	+++	4,33E-05	+
HEART	6,93E-04	++	2,599E-06	+/-	1,33E-02	++++	9,93E-06	+/-
KIDNEY	3,16E-04	++	5,304E-07	-	1,79E-02	++++	2,92E-06	+/-
LIVER	1,82E-02	++++	9,122E-07	-	2,88E-03	+++	4,35E-06	+/-
LUNG	2,52E-03	+++	1,308E-06	+/-	1,59E-02	++++	4,93E-05	+
OVARY	1,22E-04	++	6,971E-07	-	1,27E-02	++++	3,55E-06	+/-
PLACENTA	3,48E-03	+++	8,064E-06	+/-	2,68E-03	+++	3,27E-06	+/-
PROSTATE	8,90E-04	++	1,999E-06	+/-	8,18E-03	+++	6,58E-06	+/-
SKEL.MUSCLE	2,77E-03	+++	5,986E-06	+/-	9,65E-03	+++	2,04E-05	+
SMALL INTEST.	6,56E-03	+++	1,166E-06	+/-	5,12E-03	+++	1,41E-04	++
SPLEEN	3,21E-03	+++	8,921E-07	-	3,40E-03	+++	1,00E-04	++
TESTIS	3,49E-01	+++++	3,41E-04	++	2,07E-01	+++++	3,22E-04	++
THYMUS	1,18E-04	++	8,322E-07	-	5,74E-03	+++	1,69E-03	+++
THYROID	1,11E-03	+++	1,327E-06	+/-	3,75E-02	++++	4,89E-06	+/-
TRACHEA	2,30E-04	++	1,17E-04	++	7,64E-02	++++	3,79E-05	+
UTERUS	1,60E-03	+++	2,361E-06	+/-	9,01E-03	+++	6,30E-06	+/-

A

	Cancer-testis antigens							
	BORIS		MAGE-A3		Sp17		hTERT	
	MNE	Level	MNE	Level	MNE	Level	MNE	Level
SKOV-3	6,90E-04	++	1,49E-05	+	3,03E-02	++++	5,66E-04	++
OVCAR-3	7,45E-03	+++	8,98E-06	+/-	2,50E-02	++++	1,82E+00	++++++
RL-95-2	3,72E-04	++	3,56E-05	+	1,40E-02	++++	1,92E-03	+++
HEC-1	2,04E-03	+++	3,33E-05	+	2,68E-02	++++	4,90E-04	++
SK-UT-1	ND	ND	5,23E-04	++	6,28E-02	++++	2,97E-04	++

B

Figure 2: TAA expression in normal tissues.
A panel of normal human tissues was assayed for expression of BORIS, MAGE-A3, Sp17 and hTERT by quantitative RT-PCR. Values represent the mean normalized expression of the TAA normalized to β-actin and β-glucuronidase. Cells are color-coded from low expression (green) to high expression (red).B. TAA analysis in commercially available cell lines.

TAA expression in uterine cancer at mRNA level

When looking at the mRNA expression levels of BORIS in our sample collection, we noted that BORIS was detectable in 60 % of healthy controls, 50 % of endometrial carcinomas and 92 % of uterine sarcomas. Furthermore, we observed BORIS overexpression (calculated as > 2-fold increase compared with the mean expression in healthy controls) in 26 % of endometrial carcinomas and 62 % of uterine sarcomas (Table 3). The expression seems slightly, yet not significantly increased in uterine sarcoma (US) compared with endometrial carcinoma (EMCAR; Fig. 3A). Due to a fairly small amount of samples tested for this antigen, no analysis concerning clinicopathological features was performed. All clinicopathological features of the samples that were currently analyzed are summarized in Table 4.

Table 3: Evaluation of TAA expression in patient biopsies by qRT-PCR

TAA	Samples	Healthy controls	Endometrial carcinoma	Uterine sarcoma
BORIS	# samples tested (%)	9 (100)	49 (100)	15 (100)
	# evaluable samples (%)[1]	5 (56)	43 (88)	13 (87)
	# undetectable samples (%)[2]	2 (40)	21 (50)	1 (8)
	% > 2-fold change[3] (%)		11 (50)	8 (67)
MAGE-A3	# samples tested (%)	12 (100)	71 (100)	35 (100)
	# evaluable samples (%)[1]	10 (83)	70 (99)	34 (97)
	# undetectable samples (%)[2]	0 (0)	7 (10)	3 (9)
	% > 2-fold change[3]		7 (11)	3 (9)
Sp17	# samples tested (%)	12 (100)	71 (100)	34 (100)
	# evaluable samples (%)[1]	10 (83)	70 (99)	34 (100)
	# undetectable samples (%)[2]	0 (0)	0 (0)	0 (0)
	% > 2-fold change[3]		14 (20)	1 (3)
hTERT	# samples tested (%)	9 (100)	70 (100)	34 (100)
	# evaluable samples (%)[1]	9 (100)	70 (100)	33 (97)
	# undetectable samples (%)[2]	6 (67)	0 (0)	26 (79)
	% > 2-fold change[3]		50 (71)	16 (59)

[1] A sample is considered evaluable if the Ct of the reference genes is below 30. [2] A sample is considered undetectable when no Ct value could be measured (i.e. Ct > 40). [3] Numbers are expressed as > 2-fold change compared to the mean expression of normal endometrium.

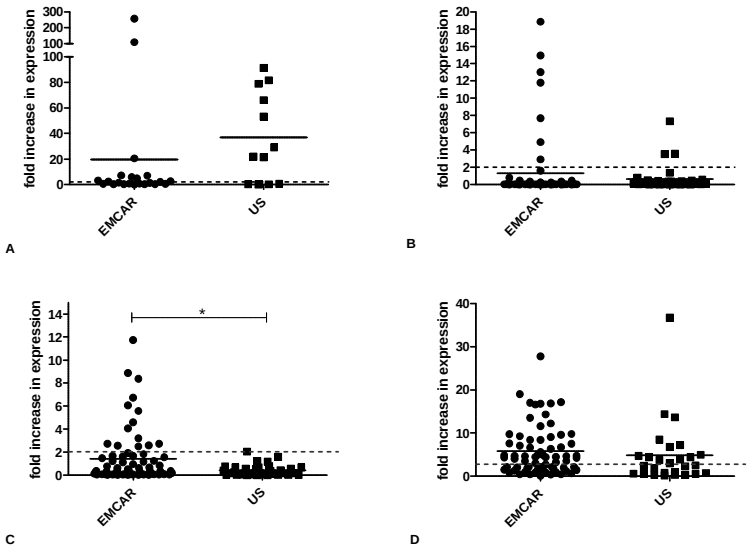


Figure 3: mRNA expression of TAA in uterine tumors. A. BORIS expression. B. MAGE-A3. C. Sp17. D. hTERT. Results are expressed as fold change compared to the mean normalized expression of normal endometrium. Overexpression is defined as at least two-fold increase compared to normal endometrium (dashed line). After verifying normal distribution, all comparisons were analyzed using the Mann Whitney test or an unpaired t test, depending on distribution outcome.

MAGE-A3 could be detected in almost all samples (100 % of controls, 90 % EMCAR and 91 % US) and was overexpressed at mRNA level in 10 % of EMCAR samples and 9 % of US samples (Table 3, Fig. 3B). No differences in MAGE-A3 expression were observed among the different FIGO stages of EMCAR, nor between the different histological subtypes or grade of differentiation of EMCAR or histological subtypes of US (data not shown).

Sp17 transcripts could be detected in all samples. Overexpression of Sp17 mRNA was found in 20 % of samples of the EMCAR population and in 3 % of uterine sarcomas (Table 3). It was significantly up-regulated in EMCAR compared with US ($p = 0.0273$, Fig. 3C). In addition, a significant down-regulation in type II EMCAR compared with type I EMCAR was found ($p = 0.0206$), indicating potential down-regulation of Sp17 with increased aggressiveness of tumors (data not shown).
We noted that hTERT was detectable in 33 % of control samples, 100 % of EMCAR and 21 % of US samples. hTERT mRNA was overexpressed in 71 % and 48 % of EMCAR and US, respectively (Table 3, Fig. 3D). When comparing hTERT expression in EMCAR according to FIGO stage, hTERT was up-regulated in stages II ($p < 0.01$), III ($p < 0.01$) and IV ($p < 0.05$) compared to stage I (data not shown).

Table 4: Clinical characteristics samples qRT-PCR analysis

Tumor type	**EMCAR**			**US**		
Tumor-associated antigen	BORIS	Sp17/ MAGE-A3	hTERT	BORIS	Sp17/hTERT	MAGE-A3
Characteristics						
Total number of selected patients	49	71	70	15	34	35
FIGO stage EMCAR (number of tumors)						
I	24	34	35			
II	8	11	10			
III	11	14	14			
IV	6	11	11			
Histological grade EMCAR						
1	17	24	24			
2	5	14	14			
3	14	22	22			
Unknown	13	11	10			
Histological subtype (number of tumors)						
Endometrioid	34	48	48			
Serous	8	12	11			
Clear cell	3	3	3			
Mixed	4	8	8			
Carcinosarcoma				6	11	11
Leiomyosarcoma				4	11	11
Rhabdomyosarcoma				1	2	2
Endometrial stromal sarcoma				4	7	8
Adenosarcoma				0	1	1
Mixed				0	1	1
Unknown				0	1	1

TAA expression in normal human tissues at protein level

Protein analysis was done only for hTERT and MUC1. For the other antigens, no optimal specific antibody was available. For MAGE-A3, there is no commercially available antibody that is specific for the detection of MAGE-A3. TAA expression in normal tissues was evaluated using a TMA containing 22 different normal tissues. We found that 42.9 % expressed MUC1. As expected, expression was predominantly found in epithelial cells. In addition, 57.6 % of tissues expressed hTERT. The tissues that express each of the investigated antigens and the respective scores are summarized in Table 5.

Table 5: TAA protein expression in normal tissues. Numbers indicate given scores according to the IHC scoring system described in Materials and methods section.

Tissue	MUC1	hTERT	Tissue	MUC1	hTERT
Adrenal gland	0	0	Liver	0	4
Urinary bladder	4	3	Lung	4	0
Breast	0	1	Ovary	N/A	N/A
Cerebellum	0	1	Pancreas	3	3
Brain	0	3	Prostate	0	3
Esophagus	3	3	Salivary gland	3	3
Stomach	1	0	Skin	1	0
Small intestine	0	3	Testis	0	0
Colon	1	1	Thyroid	0	1
Rectum	0	0	Cervix	0	1
Kidney	4	3	Endometrium	N/A	N/A

TAA expression in uterine cancer at protein level

For protein expression analysis of MUC1 and hTERT, a broad spectrum of paraffin-embedded tissue samples were analyzed. All clinicopathological characteristics are summarized in Table 6. In addition, in order to obtain an idea on the expression of a certain antigen in the tumor as a whole, specimens taken at different locations in the tumor, to a maximum of 3 different sites, were analyzed if possible. The total number of tumors that were analyzed for each of the antigens is summarized in Table 7. Microscopic analysis of the tissue samples showed that for MUC1, 96.6 % of the pooled EMCAR population expressed the antigen. In comparison with normal endometrium, primary tumors ($p < 0.05$) as well as recurrent tumors ($p < 0.01$) and metastatic lesions ($p < 0.01$) show a significant up-regulation (Fig. 4A) based on slide scoring. In addition, 43 % of the US samples was also positive for this tumor antigen. For this type of tumors, the metastatic lesions show a significant up-regulation of MUC1 in comparison to the primary and recurrent tumors ($p < 0.05$; Fig. 4A). This difference is most likely due to the high total amount of carcinosarcomas and endometrial stromal sarcomas in the total population of metastases (9/13). However, in the recurrent tumor population 8/13 tumors have either histological subtype, indicating that MUC1 is indeed expressed at the highest level in metastatic lesions.

Table 6: Clinical characteristics samples IHC analysis.

Tumor type	**EMCAR**		**US**	
Tumor-asscociated antigen	MUC1	hTERT	MUC1	hTERT
Characteristics				
Total number of patients	62	48	60	47
Total number of tumors	70	52	65	52
Number of patients				
Primary	40	27	37	24
Recurrence	8	11	10	10
Metastasis	6	7	10	10
Primary + recurrence	4	0	1	1
Primary + metastasis	4	3	1	1
Metastasis+ recurrence	0	0	1	1
FIGO stage pr EMCAR[1] (number of tumors)				
I	17	10	N/A	N/A
II	6	4	N/A	N/A
III	11	7	N/A	N/A
IV	14	9	N/A	N/A
Histological grade EMCAR[2] (number of tumors)				
1	15	7	N/A	N/A
2	6	3	N/A	N/A
3	39	32	N/A	N/A
Unknown	9	9	N/A	N/A
Histological subtype[3] (number of tumors)				
Endometrioid	29	17	N/A	N/A
Serous	24	21	N/A	N/A
Clear cell	11	9	19	18
Mixed	3	2	17	13
Carcinosarcoma[4]	2	2	9	7
Leiomyosarcoma			15	10
Rhabdomyosarcoma			5	4

[1] Only primary tumors included; [2] All tumors included; [3] All tumors included; [4] Only carinomatous tumor part included in EMCAR evaluation.

Table 7: Evaluation of TAA expression in patient biopsies by IHC.

Tumor type	**MUC1**		**hTERT**	
	# samples	**% positive**	**# samples**	**% positive**
Normal tissue	17	**82.4**	14	**92.8**
Primary carcinoma	48	**91.6**	30	**66.7**
Recurrent carcinoma	11	**100**	10	**90**
Metastatic carcinoma	10	**90**	9	**66.7**
Primary sarcoma	39	**33.3**	22	**50**
Recurrent sarcoma	15	**26.7**	12	**41.7**
Metastatic sarcoma	13	**69.2**	13	**76.9**

The protein data for hTERT showed positivity in 75.6 % of EMCAR samples and in 59.3 % of US samples. Remarkably, the antigen was significantly up-regulated in normal endometria in comparison to primary EMCAR and metastatic EMCAR locations ($p < 0.05$) as well as primary and recurrent US ($p < 0.01$, Fig. 5B).

For all the currently described antigens, no sub-analysis was done to determine differences in expression according to clinicopathological characteristics, due to too small sample sizes to perform sound analyses.

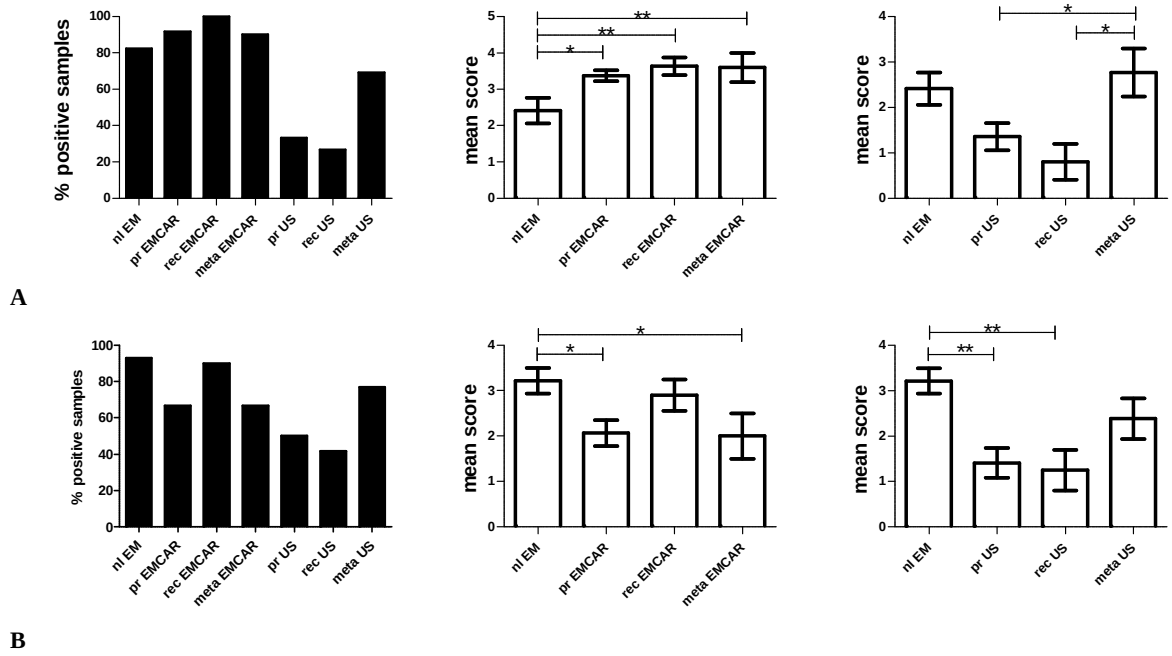


Figure 4: Expression levels of TAA by IHC. *Left panel:* overall percentage of positive biopsies. *Middle panel:* TAA expression levels in normal endometrium and EMCAR samples. *Right panel:* TAA expression levels in normal endometrium and US samples. A. MUC1 expression. B. hTERT levels. nl EM: normal endometrium; pr EMCAR: primary endometrial carcinoma; rec EMCAR: recurrent endometrial carcinoma; meta EMCAR: metastasis endometrial carcinoma; pr US: primary uterine sarcoma; rec US: recurrent uterine sarcoma; meta US: metastasis uterine sarcoma. All data were analyzed using the non-parametrical Kruskal-Wallis test, followed by the Dunn's Multiple Comparison Test.

Endogenous T cell responses to TAA

MAGE-A3, MUC1 and Sp17 were selected for further assessment of immunogenicity by determining the presence of endogenous TAA-specific T cells after stimulation with overlapping peptides covering the whole TAA. Stimulation with peptide pools covering the viral antigens CMVpp65, EBV BZLF1 and Influenza Matrix Protein 1 were included as a control for assessing immune competence. CD137 up-regulation on $CD4^+$ and $CD8^+$ T cells after *in vitro* culture of PBMC with the indicated peptide pools was measured. The clinical characteristics of the patients included in this analysis are shown in Table 7.

Virus-specific immunity as determined by CD137 up-regulation on either $CD4^+$ or $CD8^+$ T cells upon stimulation with at least 1 of the 3 viral antigens was observed in 7/9 (78%) of benign controls, 10/10 (100%) of EMCAR patients and 8/10 (80%) of US patients (data not shown). In patients that did not mount a viral response, no TAA-specific T cells could be measured (data not shown).

MAGE-A3 specific T cells could be measured in 1/9 controls for $CD4^+$ T cells and 1/9 controls for $CD8^+$ T cells. For EMCAR patients, MAGE-A3 specific $CD4^+$ and $CD8^+$ T cells were observed in 2/10 and 1/10 patients respectively, while for US patients MAGE-A3 reactivity could be noted in 4/10 patients for $CD4^+$ T cells and in 3/10 patients for $CD8^+$ T cells (Fig. 5A).
MUC1 specific $CD4^+$ and $CD8^+$ T cells were present in 2/9 and 1/9 controls respectively. For EMCAR patients, MUC1 responsiveness could only be observed in $CD4^+$ T cells in 3/10 patients. In US, MUC1 specificity was measured in 1/10 patients for $CD4^+$ T cells and in 2/10 patients for $CD8^+$ T cells (Fig. 5B).

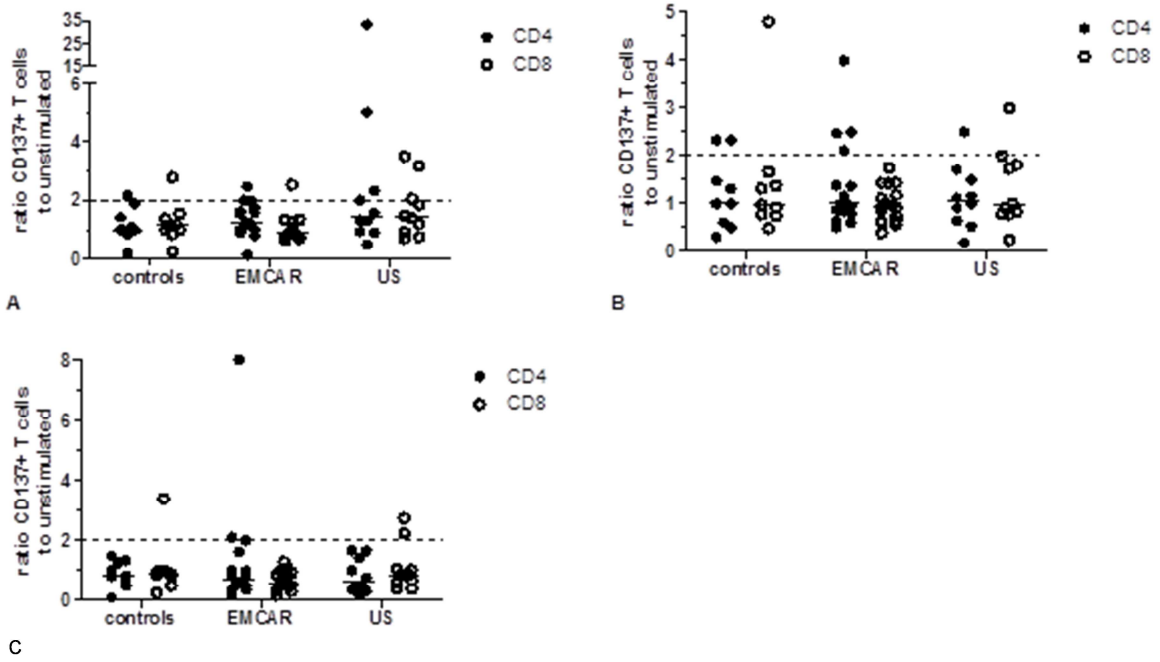


Figure 5: Analysis of T cell responses. A. $CD4^+$ and $CD8^+$ responses against MAGE-A3. B. $CD4^+$ and $CD8^+$ responses against MUC1. C. $CD4^+$ and $CD8^+$ responses against Sp17.

For Sp17, control patients only showed reactivity in 1/9 patients for $CD8^+$ T cells. In EMCAR, Sp17 specificity was seen in 2/10 patients for $CD4^+$ T cells, while Sp17 specific T cells could be noted in 2/10 US patients for $CD8^+$ T cells (Fig. 5C).
Altogether, endogenous T cells specific to any of the tested TAA were only detected in a minority of patients. For some of the tested patients, we investigated whether levels of TAA specific T cells correlate with TAA expression levels in the tumor but we could not note a correlation for any TAA (data not shown).

Table 7: Clinical characteristics samples T cell responses.

Tumor type	**Healthy/ benign**	**EMCAR**	**US**
Total number of selected patients	9	16	10
Healthy	0	N/A	N/A
Benign	9	N/A	N/A
FIGO stage			
I	N/A	9	2
II	N/A	2	0
III	N/A	1	0
IV	N/A	3	6
Unknown	N/A	1	2
Histological grade			
1	N/A	6	2
2	N/A	2	0
3	N/A	8	5
Unknown	N/A	0	3
Histological subtype			
Endometrioid	N/A	11	N/A
Serous	N/A	3	N/A
Clear cell	N/A	1	N/A
Mixed	N/A	1	N/A
Carcinosarcoma	N/A	N/A	2
Leiomyosarcoma	N/A	N/A	3
Rhabdomyosarcoma	N/A	N/A	1
Endometrial stromal sarcoma	N/A	N/A	2
Other	N/A	N/A	1
Unknown	N/A	N/A	1

DISCUSSION

We have currently validated the presence of 5 different antigens in uterine tumors, comprising both endometrial carcinoma and uterine sarcoma. To our knowledge, this is the first study to describe the presence of these antigens in uterine sarcoma, with the exception of BORIS [23]. This TAA has emerged fairly recent and has been investigated in several tumor types [24]. BORIS was described in uterine cancer by Risinger et al. [23]. These authors describe BORIS expression in 77 % of analyzed EMCAR samples as well as BORIS expression in 77 % uterine mesodermal tumors (carcinosarcomas). Analysis of BORIS in the current EMCAR samples showed BORIS mRNA expression in 60% of normal endometria and 50% of EMCAR, with an overexpression in 26% of the EMCAR samples in which BORIS could be detected. Our results do not correspond to the results of Risinger et al. Using a different primer set, they have shown expression in 77% of EMCAR and no expression in normal endometrium. A recent study concerning the expression of BORIS and its paralogue CTCF in EMCAR showed that BORIS RNA was significantly increased in non-endometrioid tumors compared with endometrioid tumors [25]. In addition, BORIS levels increased during cancer progression and were associated with aggressive tumors. Importantly, an association between BORIS expression and worse 5- year disease-free survival was found.

In contradiction to previously published results by Chitale et al. [26], who found MAGE-A3 to be present in 20% of 130 analyzed endometrial cancers, we found MAGE-A3 to be present in 90% of the analyzed tumor biopsies, overexpressed compared to normal endometrium in only 10 % of cases. Chitale et al. performed their analysis based on a TMA applying a threshold for positivity of protein expression in > 50% of tumor cells, while we currently analyzed the presence of mRNA encoding for MAGE-A3, which allows for the detection of lower levels of TAA expression.
Sp17 analysis revealed that the transcript is present in 100% of the currently analyzed EMCAR samples. The percentage of tumors expressing Sp17 is higher than found by Li et al. [27]. However, Li et al. analyzed Sp17 protein expression, which may explain the discrepancy. It may indicate that, even though present as mRNA, it may possibly not be translated into a functional protein in the majority of cases. Next to its presence in uterine tumors, Sp17 has also been shown in ovarian tumors [28, 29]. Sp17 RNA was detected in 83% of ovarian tumors. In addition, Sp17 has been shown to be related to chemoresistance in ovarian cancer.
MUC1 expression was seen in the vast majority of all analyzed samples of both normal endometrium and EMCAR. Our results showed a higher percentage of positive tumors with respect to previously published data [30]. We did not expect to find great differences between the percentage of biopsies expressing MUC1 when comparing normal endometria to EMCAR samples since wild-type MUC1 is expressed in normal epithelium, which was also shown by the TMA analysis of normal tissues. MUC1 as tumor antigen differs from the wild-type by a hypoglycosylation and the antibody directed against MUC1 that was used in the current immunohistochemical staining detects both the wild-type and the truncated form of MUC1. MUC1 has been described to play a role in tissue invasion of tumor cells and the formation of metastases [31-33]. In our data, we show that there is an increase in MUC1 expression level when comparing normal endometrial samples to primary carcinoma samples as well as to recurrent EMCAR and metastatic lesions, further supporting these data.
Telomerase was chosen as target to evaluate due to their role in tumor cell proliferation and survival. hTERT was found to be present in its RNA form in 100% of analyzed EMCAR samples. These results are comparable to previously published results, showing its expression in 78% [34] and 92% [35] of EMCAR samples. The current mRNA and protein data for hTERT expression do not correspond entirely. Although some of the expression levels we found in normal tissue were similar to previously described expression levels [36], we found high hTERT expression in brain, kidney, prostate, pancreas and liver, and a negative result for testis tissue. This is quite unexpected and in contradiction to the results of Hiyama et al., and Kyo et al. [34, 36]. In addition, we found hTERT protein to be higher in normal endometrial samples compared to EMCAR and US. This phenomenon cannot be explained solely by hTERT expression in proliferative endometrium, as we also found clear expression in atrophic endometrium, contradicting previous results [37]. These results led us to suspect that the antibody that was used for the immunohistochemistry is likely not ideal. mRNA data showed that hTERT is overexpressed in 71% of tumors compared with normal endometrium and we found an up-regulation in more advanced tumors, supporting the role of hTERT in immortalization and tumor development.

To our knowledge, none of the currently analyzed TAA has been described in uterine sarcoma before, with the exception of BORIS, as described above.
Like in EMCAR, we found expression of the analyzed cancer testis antigens in US samples. BORIS, MAGE-A3 and Sp17 were found to be present in the vast majority to all of the analyzed biopsies. In US samples, we found MUC1 expression in 43% of tumors. This is not unexpected since MUC1 is an

epithelial antigen and sarcomas are of mesenchymal origin. Our current tumor population, however, also contains malignant mixed mesodermal tumors, also known as carcinosarcomas, endometrial stromal sarcomas, and adenosarcomas, which all contain an epithelial component where MUC1 is present. Although the majority of the positive sarcomas are attributable to these two types of tumors, we did find other sarcomatous tumors to be positive for MUC1.
hTERT was expressed both as mRNA transcript and protein in sarcoma samples. In addition, we found a significant down-regulation of hTERT in primary and recurrent US in comparison to normal endometrium.

The immunogenicity of an antigen, i.e. the ability to elicit an immune response is a key characteristic in order for the antigen to be eligible as a target for immunotherapy. Spontaneous T cell responses against several TAA, mainly melanoma-associated antigens, have been described in several tumor types, both solid tumors and hematological tumors [38]. To our knowledge, spontaneous T cell responses to any of the tested TAA in this study have not been reported to date in either endometrial carcinoma or uterine sarcoma.
We found endogenous T cells reactive to any of the tested TAA in only a minority of patients. Moreover, for MAGE-A3, MUC1 and Sp17, comparable levels of TAA-specific T cells were found in patients with benign gynecological afflictions.
Inokuma et al previously showed low level MAGE-A3 T cell responses in healthy females which were increased in breast cancer patients [39], which corroborates our finding of spontaneous MAGE-A3 specific T cells in patients with benign diseases. Our currently analyzed patient population is however too small to evaluate whether these responses are increased in endometrial carcinoma or uterine sarcoma.
The presence of pre-existing MUC1 specific T cell immunity in both healthy donors as well as breast cancer patients has been described by Gückel et al, which corroborates our findings of MUC1 reactive T cells in patients with benign diseases [40].
Spontaneous Sp17 specific T cells have, to our knowledge, not been described to date, neither in healthy donors, nor in cancer patients. The group of Chiriva-Internati, however, have shown that it is possible to generate Sp17 specific T cells after 4 rounds of *in vitro* stimulations with Sp17-pulsed DC in patients with ovarian cancer and multiple myeloma and in healthy donors [41-43]. These data suggest the presence of low levels of Sp17 specific T cells in the circulation of both healthy donors and cancer patients.

In conclusion, the current data collectively validate the presence of the selected TAA in endometrial cancer and indicate the presence of those antigens in uterine sarcoma. In addition, spontaneous T cell responses could be detected in patient blood, indicating the immunogenicity of these antigens. Based on the combined analysis of TAA expression data and the occurrence of TAA-specific T cells, MUC1 shows the best profile for future application in immunotherapeutic studies, along with the previously validated survivin (Vanderstraeten A et al., submitted) These data are a further indication of their possible applicability in immunotherapeutic regimens to treat uterine cancer patients.

REFERENCES

1. Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, et al. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. Clin Cancer Res. 2009;15(17):5323-37.
2. Vacchelli E, Vitale I, Eggermont A, Fridman WH, Fucikova J, Cremer I, et al. Trial watch: Dendritic cell-based interventions for cancer therapy. Oncoimmunology. 2013;2(10):e25771.
3. Carrasco J, Van Pel A, Neyns B, Lethe B, Brasseur F, Renkvist N, et al. Vaccination of a melanoma patient with mature dendritic cells pulsed with MAGE-3 peptides triggers the activity of nonvaccine anti-tumor cells. J Immunol. 2008;180(5):3585-93.
4. Dillman RO, Selvan SR, Schiltz PM, McClay EF, Barth NM, DePriest C, et al. Phase II trial of dendritic cells loaded with antigens from self-renewing, proliferating autologous tumor cells as patient-specific antitumor vaccines in patients with metastatic melanoma: final report. Cancer Biother Radiopharm. 2009;24(3):311-9.
5. Ohno S, Kyo S, Myojo S, Dohi S, Ishizaki J, Miyamoto K, et al. Wilms' tumor 1 (WT1) peptide immunotherapy for gynecological malignancy. Anticancer Res. 2009;29(11):4779-84.
6. Wilgenhof S, Van Nuffel AM, Corthals J, Heirman C, Tuyaerts S, Benteyn D, et al. Therapeutic vaccination with an autologous mRNA electroporated dendritic cell vaccine in patients with advanced melanoma. J Immunother. 2011;34(5):448-56.
7. Sampson JH, Heimberger AB, Archer GE, Aldape KD, Friedman AH, Friedman HS, et al. Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. J Clin Oncol. 2010;28(31):4722-9.
8. Wheeler CJ, Black KL, Liu G, Mazer M, Zhang XX, Pepkowitz S, et al. Vaccination elicits correlated immune and clinical responses in glioblastoma multiforme patients. Cancer Res. 2008;68(14):5955-64.
9. Herbert N, Haferkamp A, Schmitz-Winnenthal HF, Zoller M. Concomitant tumor and autoantigen vaccination supports renal cell carcinoma rejection. J Immunol. 2010;185(2):902-16.
10. Van Tendeloo VF, Van de Velde A, Van Driessche A, Cools N, Anguille S, Ladell K, et al. Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. Proc Natl Acad Sci U S A. 2010;107(31):13824-9.
11. Bijen CB, Bantema-Joppe EJ, de Jong RA, Leffers N, Mourits MJ, Eggink HF, et al. The prognostic role of classical and nonclassical MHC class I expression in endometrial cancer. Int J Cancer. 2010;126(6):1417-27.
12. Vanderstraeten A, Tuyaerts S, Amant F. The immune system in the normal uterus and its implication in uterine tumor development (submitted).
13. Coosemans A, Wolfl M, Berneman ZN, Van Tendeloo V, Vergote I, Amant F, et al. Immunological response after therapeutic vaccination with WT1 mRNA-loaded dendritic cells in end-stage endometrial carcinoma. Anticancer Res. 2010;30(9):3709-14.
14. Coosemans A, Vanderstraeten A, Tuyaerts S, Verschuere T, Moerman P, Berneman ZN, et al. Wilms' Tumor Gene 1 (WT1)--loaded dendritic cell immunotherapy in patients with uterine tumors: a phase I/II clinical trial. Anticancer Res. 2013;33(12):5495-500.
15. Hernando JJ, Park TW, Kubler K, Offergeld R, Schlebusch H, Bauknecht T. Vaccination with autologous tumour antigen-pulsed dendritic cells in advanced gynaecological malignancies: clinical and immunological evaluation of a phase I trial. Cancer Immunol Immunother. 2002;51(1):45-52.
16. Santin AD, Bellone S, Ravaggi A, Roman JJ, Pecorelli S, Parham GP, et al. Induction of tumour-specific CD8(+) cytotoxic T lymphocytes by tumour lysate-pulsed autologous dendritic cells in patients with uterine serous papillary cancer. Br J Cancer. 2002;86(1):151-7.
17. Costa FF, Le Blanc K, Brodin B. Concise review: cancer/testis antigens, stem cells, and cancer. Stem Cells. 2007;25(3):707-11.

18. D'Arcy V, Pore N, Docquier F, Abdullaev ZK, Chernukhin I, Kita GX, et al. BORIS, a paralogue of the transcription factor, CTCF, is aberrantly expressed in breast tumours. Br J Cancer. 2008;98(3):571-9.
19. Hines WC, Bazarov AV, Mukhopadhyay R, Yaswen P. BORIS (CTCFL) is not expressed in most human breast cell lines and high grade breast carcinomas. PLoS One. 2010;5(3):e9738.
20. Lladser A, Sanhueza C, Kiessling R, Quest AF. Is survivin the potential Achilles' heel of cancer? Advances in cancer research. 2011;111:1-37.
21. Patel KP, Vonderheide RH. Telomerase as a tumor-associated antigen for cancer immunotherapy. Cytotechnology. 2004;45(1-2):91-9.
22. Beatson RE, Taylor-Papadimitriou J, Burchell JM. MUC1 immunotherapy. Immunotherapy. 2010;2(3):305-27.
23. Risinger JI, Chandramouli GV, Maxwell GL, Custer M, Pack S, Loukinov D, et al. Global expression analysis of cancer/testis genes in uterine cancers reveals a high incidence of BORIS expression. Clin Cancer Res. 2007;13(6):1713-9.
24. Martin-Kleiner I. BORIS in human cancers -- a review. European journal of cancer. 2012;48(6):929-35.
25. Hoivik EA, Kusonmano K, Halle MK, Berg A, Wik E, Werner HM, et al. Hypomethylation of the CTCFL/BORIS promoter and aberrant expression during endometrial cancer progression suggests a role as an Epi-driver gene. Oncotarget. 2014.
26. Chitale DA, Jungbluth AA, Marshall DS, Leitao MM, Hedvat CV, Kolb D, et al. Expression of cancer-testis antigens in endometrial carcinomas using a tissue microarray. Mod Pathol. 2005;18(1):119-26.
27. Li FQ, Liu Q, Han YL, Wu B, Yin HL. Sperm protein 17 is highly expressed in endometrial and cervical cancers. BMC Cancer. 2010;10:429.
28. Nakazato T, Kanuma T, Tamura T, Faried LS, Aoki H, Minegishi T. Sperm protein 17 influences the tissue-specific malignancy of clear cell adenocarcinoma in human epithelial ovarian cancer. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society. 2007;17(2):426-32.
29. Straughn JM, Jr., Shaw DR, Guerrero A, Bhoola SM, Racelis A, Wang Z, et al. Expression of sperm protein 17 (Sp17) in ovarian cancer. Int J Cancer. 2004;108(6):805-11.
30. Sivridis E, Giatromanolaki A, Koukourakis MI, Georgiou L, Anastasiadis P. Patterns of episialin/MUC1 expression in endometrial carcinomas and prognostic relevance. Histopathology. 2002;40(1):92-100.
31. Gao J, McConnell MJ, Yu B, Li J, Balko JM, Black EP, et al. MUC1 is a downstream target of STAT3 and regulates lung cancer cell survival and invasion. Int J Oncol. 2009;35(2):337-45.
32. Suzuki Y, Sutoh M, Hatakeyama S, Mori K, Yamamoto H, Koie T, et al. MUC1 carrying core 2 O-glycans functions as a molecular shield against NK cell attack, promoting bladder tumor metastasis. Int J Oncol. 2012;40(6):1831-8.
33. Ye Q, Yan Z, Liao X, Li Y, Yang J, Sun J, et al. MUC1 induces metastasis in esophageal squamous cell carcinoma by upregulating matrix metalloproteinase 13. Lab Invest. 2011;91(5):778-87.
34. Kyo S, Kanaya T, Takakura M, Tanaka M, Inoue M. Human telomerase reverse transcriptase as a critical determinant of telomerase activity in normal and malignant endometrial tissues. Int J Cancer. 1999;80(1):60-3.
35. Lehner R, Enomoto T, McGregor JA, Shroyer AL, Haugen BR, Pugazhenthi U, et al. Quantitative analysis of telomerase hTERT mRNA and telomerase activity in endometrioid adenocarcinoma and in normal endometrium. Gynecologic oncology. 2002;84(1):120-5.
36. Hiyama E, Hiyama K, Yokoyama T, Shay JW. Immunohistochemical detection of telomerase (hTERT) protein in human cancer tissues and a subset of cells in normal tissues. Neoplasia. 2001;3(1):17-26.
37. Kyo S, Masutomi K, Maida Y, Kanaya T, Yatabe N, Nakamura M, et al. Significance of immunological detection of human telomerase reverse transcriptase: re-evaluation of expression and

localization of human telomerase reverse transcriptase. The American journal of pathology. 2003;163(3):859-67.
38. Nagorsen D, Scheibenbogen C, Marincola FM, Letsch A, Keilholz U. Natural T cell immunity against cancer. Clin Cancer Res. 2003;9(12):4296-303.
39. Inokuma M, dela Rosa C, Schmitt C, Haaland P, Siebert J, Petry D, et al. Functional T cell responses to tumor antigens in breast cancer patients have a distinct phenotype and cytokine signature. J Immunol. 2007;179(4):2627-33.
40. Guckel B, Rentzsch C, Nastke MD, Marme A, Gruber I, Stevanovic S, et al. Pre-existing T-cell immunity against mucin-1 in breast cancer patients and healthy volunteers. Journal of cancer research and clinical oncology. 2006;132(4):265-74.
41. Chiriva-Internati M, Wang Z, Salati E, Bumm K, Barlogie B, Lim SH. Sperm protein 17 (Sp17) is a suitable target for immunotherapy of multiple myeloma. Blood. 2002;100(3):961-5.
42. Chiriva-Internati M, Wang Z, Salati E, Timmins P, Lim SH. Tumor vaccine for ovarian carcinoma targeting sperm protein 17. Cancer. 2002;94(9):2447-53.
43. Chiriva-Internati M, Wang Z, Salati E, Wroblewski D, Lim SH. Successful generation of sperm protein 17 (Sp17)-specific cytotoxic T lymphocytes from normal donors: implication for tumour-specific adoptive immunotherapy following allogeneic stem cell transplantation for Sp17-positive multiple myeloma. Scandinavian journal of immunology. 2002;56(4):429-33.

Part IV Immunosuppression in uterine tumors

Part IV

Chapter 5 Mapping the immunosuppressive environment in uterine tumors: implications for immunotherapy

Vanderstraeten A, Luyten C, Verbist G, Tuyaerts S, Amant F
Cancer Immunology Immunotherapy, CII. 2014;63(6):545-57.

Mapping the immunosuppressive environment in uterine tumors: implications for immunotherapy

The major hurdle for cancer vaccines to be effective is posed by tumor immune evasion. Several common immune mechanisms and mediators are exploited by tumors to avoid immune destruction. In an attempt to shed more light on the immunosuppressive environment in uterine tumors, we analyzed the presence of PD-L1, PD-L2, B7-H4, indoleamine 2,3-dioxygenase (IDO), galectin-1, galectin-3, arginase-1 activity and myeloid-derived suppressor cell (MDSC) infiltration.

IDO, PD-L1, PD-L2 and B7-H4 were analyzed by immunohistochemistry. PD-L2 was mostly expressed at low levels in these tumors. We found high IDO expression in21 % of endometrial carcinoma samples and in 14 % of uterine sarcoma samples. For PD-L1 and B7-H4 we found high expression in 92 % and 90 % of endometrial cancers respectively and in 100 % and 92 % of the sarcomas. Galectin-1 and 3 were analyzed in tissue lysates by ELISA, but we did not find an increase in both molecules in tumor lysates compared with benign tissues. We detected expression of galectin-3 by fibroblasts, immune cells and tumor cells in single-cell tumor suspensions. In addition, we noted a highly significant increase in arginase-1 activity in endometrial carcinomas compared with normal endometria, which was not the case for uterine sarcomas. Finally, we could demonstrate MDSC infiltration in fresh tumor suspensions from uterine tumors.

These results indicate that the PD-1/PD-L1 interaction and B7-H4 could be possible targets for immune intervention in uterine cancer patients as well as mediation of MDSC function. These observations are another step toward the implementation of inhibitors of immunosuppression in the treatment of uterine cancer patients.

INTRODUCTION

Currently used immunotherapeutic regimens often result in immunological responses, such as antigen-specific T cell responses, but are all too often not accompanied by a correlating clinical response. One of the reasons for the lack of consistent clinical responses is thought to be attributable to immune escape exerted by the tumor. Various mechanisms of immune evasion by tumors have been described, but the picture in uterine tumors is still enigmatic. By increasing our knowledge on the involvement of these immune escape pathways in uterine tumors, we aim to identify new treatment options for this cancer type for which not much progress has been made in recent years. One of the possible immune escape mechanisms are the so-called immune checkpoints. Immune checkpoints, some of which are briefly outlined below, comprise an array of pathways which the immune system occupies to maintain self-tolerance. Besides their natural function in the prevention of among others autoimmunity, these molecules also play an important role in antitumor immunity, more specifically in the prevention/blockage thereof. The two checkpoints most focused on are CTLA-4 and PD-1. Several inhibitors of these molecules are currently in clinical development and being analyzed in both the preclinical setting as well as in clinical trials, with the aim of improving treatment outcome and survival in cancer patients [1-4].

PD-L1 and PD-L2, both members of the B7-CD28 family, are ligands for the death receptor Programmed Death Receptor 1 (PD-1), which play an important role in the central T cell tolerance

during T cell development [5]. PD-L1 is expressed in a variety of tissues, such as placenta, heart and spleen cells as well as islets and leukocytes [5, 6]. In tumors, PD-L1 expression has been abundantly detected and is often associated with a poor prognosis [7-9]. The expression pattern of PD-L2 on the other hand is much more restricted. It is expressed mainly on dendritic cells and macrophages [6], but expression can be induced on several other immune and non-immune cells depending on environmental stimuli [10]. PD-L2 expression was shown in different tumor types, although mostly in a minority of patients [11-13]. PD-1/PD-L1 interactions have been shown to inhibit antigen-experienced T cells in the periphery, thereby protecting normal tissues from immune destruction [3]. PD-L2 on the other hand is regulated by Th2 cytokines and may itself possibly be involved in the modulation of Th2 responses [10].

B7-H4 mRNA is abundantly detected in human somatic tissues, but protein expression on tissues is limited [14]. On the contrary, B7-H4 protein expression has been found in many different types of human tumors [14] and soluble B7-H4 can be detected in the serum of cancer patients [15, 16]. B7-H4 has been shown to play a role in the inhibition of activation, proliferation and clonal expansion of both $CD4^+$ and $CD8^+$ T cells [14, 17].

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme mediating the rate-limiting step of tryptophan catabolism along the kynurenine pathway. IDO mediates “metabolic immune regulation” by depriving T cells of tryptophan and by the production of toxic metabolites, both leading to inhibition of T cell proliferation and induction of T cell death. Furthermore, IDO can convert naïve T cells into regulatory T cells (Treg) and enhances their activity [18]. IDO expression has been observed in a variety of cancers, and high IDO expression is associated with dismal prognosis [19].

Another family with important immunoregulatory functions is the galectin family. The current manuscript is focused on galectin-1 and galectin-3. Galectin-1 expression by tumors is associated with poor prognosis and the formation of metastasis through modulation of among others cell migration, adhesion and angiogenesis [20]. Besides its direct effects on tumor progression, galectin-1 also plays a role in tumor immune regulation by creating a bias toward a Th2 profile and activation of tolerogenic DC and IL-10 producing regulatory (Tr1) cells. Galectin-3 is involved in the differentiation and proliferation of several immune cells. It activates both lymphoid and myeloid cells, such as T cells, mast cells, monocytes and neutrophils [20]. Galectin-3 is also a negative regulator of immune cell function by controlling the anergic state of T cells [21]. Galectin-3 is capable of forming lattices with the T cell receptor complex, thereby intervening with T cell receptor signaling and subsequent T cell activation [20].

Other key players in tumor immune suppression are certain types of immune cells, such as Treg or myeloid-derived suppressor cells (MDSC). The latter are a heterogeneous population of immature myeloid cells with monocytic and/or granulocytic features. MDSC exert their suppressive function by depleting L-arginine from the tumor micro-environment via the production of arginase-1. The depletion of L-arginine subsequently causes a down-regulation of the ζ-chain of the T cell receptor, [22, 23] as well as cell cycle arrest of T cells [24]. Furthermore, MDSC produce reactive oxygen species (ROS) and nitric oxide (NO) radicals, which also capable of suppressing T cell function [25]. MDSC have been implicated in various tumors and are correlated with poor prognosis [26, 27]. MDSC levels might serve as a predictive marker for clinical outcome of oncologic treatment [25, 28].

We have used an immunohistochemical approach to validate the presence of IDO in uterine tumors, as well as to investigate the presence of PD-L1/PD-L2, to our knowledge not described in this type of tumor before, and B7-H4.

In addition, we have analyzed the expression of galectin-1 and 3 in uterine tissue samples by ELISA and confirmed galectin-3 expression on cellular subtypes in uterine tumor specimens by flow cytometry. The activity of arginase-1 in uterine tissue samples was determined, and we further provided evidence of MDSC infiltration in uterine tumor specimens by multi-parametric flow cytometry.

MATERIALS AND METHODS

Biopsy material

All biopsies were collected at the University Hospital Leuven, campus Gasthuisberg, from patients undergoing surgery at the Department of Gynecologic Oncology. When possible, multiple blocks were used from the same tumor to a maximum of three tumor blocks per tumor. The latter was done in order to obtain an idea of expression across the entire tumor. Samples of normal endometrium or myometrium were collected from patients undergoing surgery for benign afflictions.

The collection of all human samples was approved by the Institutional Review Board of the University Hospitals Leuven and written informed consent was obtained from all patients.

Immunohistochemical staining

For all stainings 4.0 µm formalin- or Bouin-fixed paraffin-embedded tissue slides were used. All slides were deparaffinized and rehydrated prior to staining. The general steps for each of the staining procedures are similar comprising the consecutive steps of endogenous alkaline phosphatase or peroxidase inhibition using 0.2N HCl or 0.5% H_2O_2 in methanol, respectively, followed by heat-induced epitope retrieval (HIER), blocking of slides to prevent aspecific antibody binding, and application of the appropriate antibodies and visualization methods. The specifications of these steps for each of the staining procedures are summarized in Table 1.

Table 1: Experimental conditions of IHC procedures.

Target	**IDO**	**PD-L1**	**PD-L2**	**B7-H4**
HIER				
Buffer	Tris-HCl + 1mM EDTA	Tris-HCl + 1mM EDTA	Tris-HCl + 1mM EDTA	citrate
Time	2h	1.5h	2h	1h
Temperature	90°C	90°C	90°C	90°C
Primary antibody				
Antibody	Ms anti Hu IDO (Chemicon)	Rb anti Hu PD-L1 (Abcam)	Ms anti Hu PD-L2 (R&D Systems)	Rb anti Hu B7-H4 (Epitomics)
Final concentration	2 µg/ml	Ready to use	0,8 µg/ml	1/600 dilution
Secondary antibody	Go anti Ms IgG-Po + APAAP complex	Envision system anti Rb-HRP	Envision system anti Ms-HRP	Envision system anti Rb-HRP
Visualization	NBT	DAB	DAB	DAB
Counterstaining	hematoxylin	hematoxylin	hematoxylin	hematoxylin

Scoring system

Before scoring the tissue slides, tumor tissue was identified using a conventionally stained HE tissue slide of the same tumor. All slides were scored with a scoring system, partially adopted from Ino et al. [29]. All slides were given two separate scores. One for the percentage of total tumor cells showing expression, divided in three different categories: 1-25; 25-50; >50 %. Each of these parameters was subsequently given a score of 1, 2 and 3 respectively. The second score was given according to the intensity of the expression: 1 (weak), 2 (moderate) and 3 (strong). Depending on the sum of the 2 scores, a final value was given. A sum of 0-1 = final score 0; 2-3 = 1; 4-5 = 2 and 6 = 3. Based on these scores, a discrimination between biopsies with high and low expression levels was made, adopted from De Jong et al. [30]. Biopsies with a total score of 0-1 are classified as low score (lo), and biopsies with a total score of 2-3 are classified as high score (hi). The intensity scores which match the visual aspect of the staining were determined for each molecule separately.

Tumor lysate preparation

150 - 200 mg of snap-frozen tissue samples were lysed by mechanical friction using magnetic stones after adding lysis buffer consisting of 250 mM Tris HCl, 750 mM NaCl, 0.5 % SDS, 2.5 % deoxycholic acid, 5 % Igepal and 0.01 % protease inhibitor cocktail (Mammalian Cell Lysis Kit; Sigma-Aldrich). After incubating the minced tissue material for 30 min at 4 °C, the lysed material was purified from debris through centrifugation. Protein content was determined using the Pierce BCA Protein Assay (Thermo Scientific) according to manufacturer's instructions.

Galectin-1 ELISA

Ninety-six well plates were coated overnight with goat anti-human galectin-1 (R&D Systems, 2 µg/ml in PBS). After blocking the wells with 1% BSA in PBS for 1 h at room temperature (RT), standards (recombinant galectin-1; range: 25-0.39 ng/ml) and samples were added for 2 h at RT. Analyses were performed on tumor lysates at a concentration of 5 mg/ml. Detection was done using a combination of goat anti-human IgG (R&D systems, 200 ng/ml) and HRP-conjugated streptavidin, followed by incubation with TMB substrate. The reaction was stopped using 2 M H_2SO_4 and OD was measured at 450 nm, with 540 nm as reference wavelength.

Galectin-3 ELISA

Galectin-3 detection was performed using a commercially available ELISA (R&D Systems) with minor modifications. For coating, mouse anti-human galectin-3(2 µg/ml) was used. The galectin-3 standard was used within the range of 4000-62.5 pg/ml. Detection was performed using mouse anti-human IgG in combination with HRP-conjugated streptavidin. All other steps are identical to galectin-1 detection.

Arginase-1 activity assay

Measurement of arginase-1 activity, through determination of the urea content in tumor lysates (at a concentration of 5 mg/ml) was performed using the QuantiChrom™ Arginase Assay Kit (Bioassay Systems) following the manufacturer's protocol.

Preparation of single-cell suspensions from tumor biopsies

Single-cell suspensions of fresh tumor biopsies were prepared by a combination of mechanical dissociation and enzymatic digestion using the Human Tumor Dissociation Kit (Miltenyi Biotec) and the GentleMACS dissociator (Miltenyi Biotec). Briefly, the protocol for soft tumors was followed, according to manufacturer's instructions. The cell suspensions were purified on a 40 µm cell strainer and counted with Türck's solution. Single-cell suspensions from tumor biopsies were either used fresh or after cryopreservation for subsequent flow cytometric analysis.

Flow cytometric analysis

Cell suspensions were first stained with a fixable viability dye (Fixable Viability Dye eFluor 506, eBioscience) for discrimination of live and dead cells. After washing with PBS, Fc receptor blocking was performed by adding a 10 % normal goat serum (Sigma-Aldrich) solution in PBS/0.5 % BSA. For single-cell tumor suspensions, red blood cell lysis was performed after the membrane staining using 1x PharmLyse (BD Pharmingen), according to manufacturer's instructions.

Galectin-3 expression by primary tumor cell lines was evaluated by staining with galectin-3-PE (BioLegend) or the appropriate isotype control, either on the membrane or intracellularly after fixation/permeabilization using the FoxP3 Staining Buffer Set (eBioscience). Acquisition was performed with a FACS Canto II flow cytometer using BD FACS DIVA software, and 3 x 10^4 cells were acquired per sample. Data analysis was done using BD FACS DIVA software. Cells were gated on FSC/SSC characteristics, and analyzed for the expression of membranous or intracellular galectin-3. For the analysis of galectin-3 expression by different cell types, we used the following antibody cocktail: CD90-FITC (BioLegend), galectin-3-PE (BioLegend), EpCAM-PerCP-Cy5.5 (BioLegend) and CD45-APC-H7 (BD Pharmingen). Intracellular expression of galectin-3 was assessed after fixation/permeabilization with the FoxP3 Staining Buffer Set (eBioscience). Acquisition was performed with a FACS Canto II flow cytometer using BD FACS DIVA software, and between 2.5 x 10^4 and 1 x 10^6 cells were acquired in the live gate per sample. Data analysis was done using BD FACS DIVA software. Cells were gated as follows: first, dead cells were gated out using the viability dye, and cells were gated based on FSC/SSC characteristics. Next, $CD90^+$ fibroblasts, $CD45^+$ immune cells and $EpCAM^+$ tumor cells (for EMCAR), or $CD45^-$ $CD90^-$ tumor cells (for US) were gated and each analyzed for the expression of membranous or intracellular galectin-3. Appropriate isotype controls were included to set the gates for the positive and negative populations.

For enumeration of MDSC, the following antibody cocktail was used: CD45-FITC (BioLegend), CD11b-PE (BioLegend), CD14-PerCP-Cy5.5 (BD Pharmingen), CD3-PE-Cy7 (BioLegend), CD19-PE-Cy7 (BioLegend), CD56-PE-Cy7 (BioLegend), CD15-APC (BioLegend), HLA-DR-APC-H7 (BD Pharmingen) and CD33-V450 (BD Horizon). Analysis of arginase-1 expression was done by replacing CD45-FITC with Arginase-1-fluorescein (R&D Systems) in the above-mentioned MDSC cocktail. For assessment of arginase-1 expression, cells were first stained for membrane markers, subsequently fixed and permeabilized using the FoxP3 Staining Buffer Set (eBioscience) and stained with arginase-1-fluorescein (R&D Systems). Acquisition was done as mentioned above for assessment of galectin-3 on tumor subpopulations. Data analysis was done using BD FACS DIVA software. The following gating strategy was used for enumeration of MDSC: first, dead cells were gated out using the viability dye, and cells were gated based on FSC/SSC characteristics, followed by CD45 positivity. Next, a gate was

defined for lineage-negative (CD3-, CD19- and CD56-negative), HLA-DR$^{low/-}$ cells. Within this population, CD11b^{+} CD14^{-} granulocytic MDSC and CD11b^{+} CD14^{+} monocytic MDSC were specified. On both cell types, the expression of CD15 and CD33 was documented. Arginase-1 expression by MDSC, was analyzed as follows: dead cells were gated out using the viability dye, and cells were gated based on FSC/SSC characteristics. Next, the lineage-negative (CD3-, CD19- and CD56-negative), HLA-DR$^{low/-}$ cell population was identified. Within this population, CD11b^{+} CD14^{-} granulocytic MDSC and CD11b^{+} CD14^{+} monocytic MDSC were defined, and the expression of arginase-1 on both subtypes was analyzed. Proper isotype controls were used to set the gates for the positive and negative populations.

Statistics

Statistical analyses were done using the GraphPad Prism 5.0 software. All scoring data were analyzed using the nonparametric Kruskal-Wallis test, followed by the Dunn's multiple comparison test. Differences in proportion were calculated using the Fischer exact test. Multiple group comparisons were performed using either ANOVA or Student's *t*-test. Comparison of survival curves was performed using the Log-Rank (Mantel-Cox) test.

RESULTS

IDO expression in uterine tumors

Patient characteristics of the IHC analysis of IDO, PD-L1, PD-L2 and B7-H4 are summarized in Table 2. IDO is present in 57 % of normal endometria (Table 3). When looking at the carcinomatous tumors, we found IDO to be present in only 36 % of the primary endometrial cancers (EMCAR). It is slightly, yet not significantly, increased in the metastatic locations (44 %). Sixty percent of the currently analyzed recurrent EMCAR samples showed IDO expression. The enzyme is present in only a minority of sarcoma samples, and the expression level is generally low in this tumor type (Fig. 1). In comparison with normal endometrium, both the percentage of positive tumors ($p < 0.01$) and the expression level is significantly lower in primary US ($p < 0.01$) and recurrent US ($p < 0.05$). IDO is present in a significantly lower proportion of metastatic US lesions compared to normal endometrium ($p < 0.05$) and at a lower expression level ($p < 0.05$).

The expression pattern of IDO was mainly cytoplasmic. In the EMCAR biopsies, in some tumors, clear apical expression could be detected (Fig. 2). In addition, IDO expressing infiltrating cells could be detected in the tumor periphery of some of the analyzed tumors, both carcinoma and sarcoma.

Twenty-one percent of primary EMCAR samples were IDO^{hi}, while for US, 14 % of primary tumors were IDO^{hi}, indicating that IDO blockade could be a useful strategy in a minority of patients.

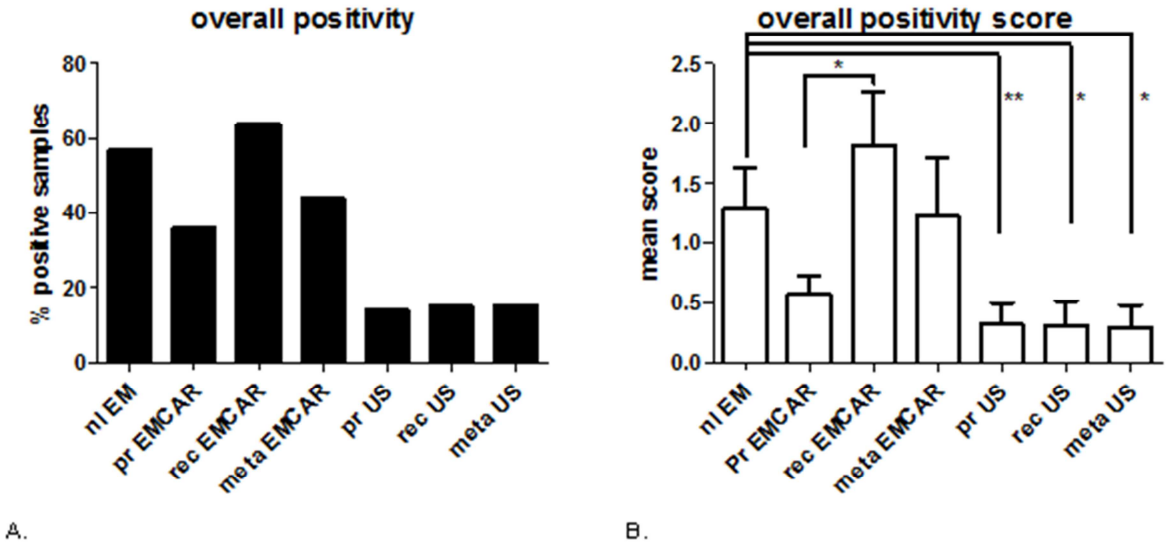


Figure 1: IDO expression levels in uterine tumors. A. Percentage of tumors/normal endometrium positive for IDO. B. Overall expression levels between groups. Statistical analysis was performed using the Kruskal-Wallis test in combination with the Dunn's Multiple Comparison Test.

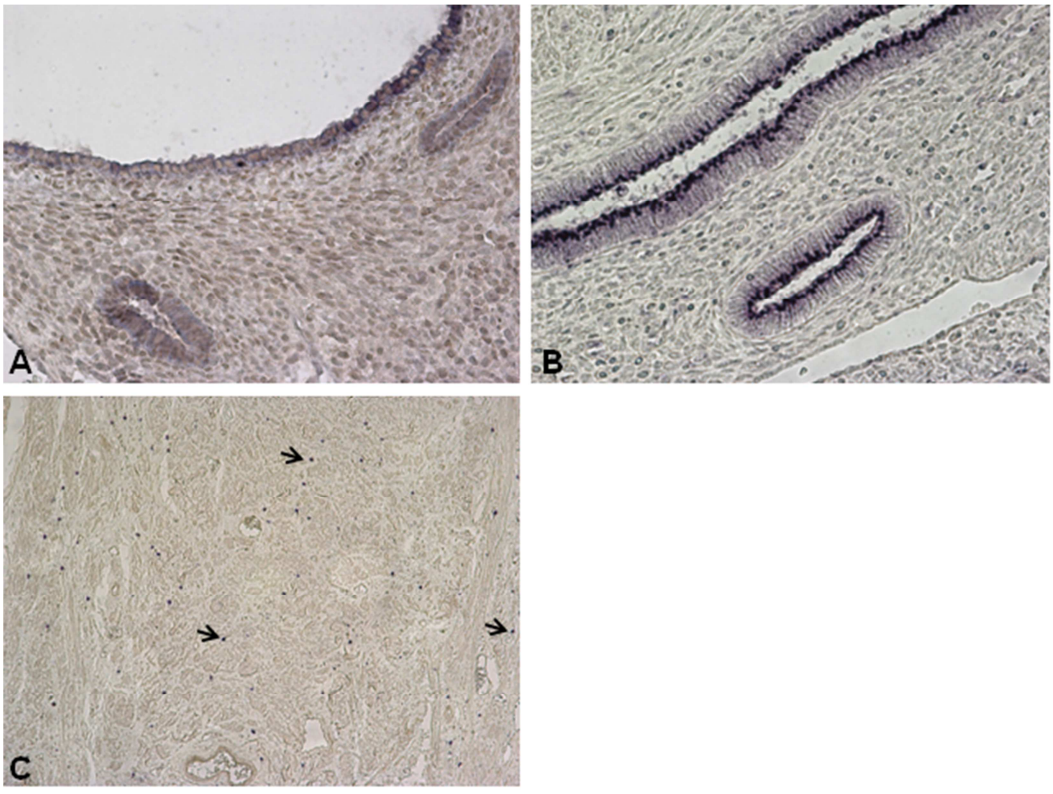


Figure 2: IDO expression in endometrial biopsies. A. normal endometrium with moderate (intensity score 2) expression levels of IDO (40x enlarged). B. normal endometrium with clear apical IDO expression at high expression level (intensity score 3, 40x enlarged). C. endometrial carcinoma biopsy with numerous infiltrating cells in the tumor stroma (black arrows, 10x enlarged).

Table 2: Clinical characteristics of biopsies used for IHC detection.

Patient characteristics	**EMCAR patients Number (%)**	**US patients Number (%)**
Total	48	47
Age (years)		
Mean	69.18	60.7
Range	43-86	26-85
Type		
Primary	27 (55.1%)	25 (53.2%)
Recurrence	11 (22.9%)	10 (21.3%)
Metastasis	7 (14.3%)	10 (21.3%)
Primary + metastasis	3 (6.1%)	1 (2.1%)
Metastasis + recurrence	0 (0.0%)	1 (2.1%)
FIGO stage[1]		
I	10 (33.3%)	N/A
II	4 (13.3%)	N/A
III	7 (23.3%)	N/A
IV	9 (30%)	N/A
Differentiation grade		
Low	7 (14.3%)	N/A
Moderate	3 (6.1%)	N/A
High	29 (59.2%)	N/A
unknown	9 (18,. 5%)	N/A
Histologic subtype		
Endometrioid	16 (32.7%)	N/A
Clear cell	8 (16.3%)	N/A
Serous papillary	20 (40.8%)	N/A
Mixed	2 (4.16%)	N/A
Carcinosarcoma[2]	2 (4.08%)	18 (38.3%)
Leiomyosarcoma	N/A	12 (25.5%)
Rhabdomyosarcoma	N/A	6 (12.8%)
Endometrial stromal sarcoma	N/A	7 (14.9%)
Adenosarcoma	N/A	4 (8.5%)

[1] FIGO stage only applicable to primary tumors. [2] Only contained carcinomatous tumor portion.

PD-L1 expression

The expression pattern for PD-L1 was cytoplasmic in all of the analyzed biopsies (Fig. 3).Overall, PD-L1 is expressed in the vast majority of the currently tested samples (Fig. 4A). We found PD-L1 expression in 81 % of normal endometria. In 4/16 of the analyzed normal endometria, PD-L1^{+} cells infiltrating the endometrium were seen. PD-L1 expression in tumors was found in 70 to 80 % of the EMCAR samples and in all of the US samples.

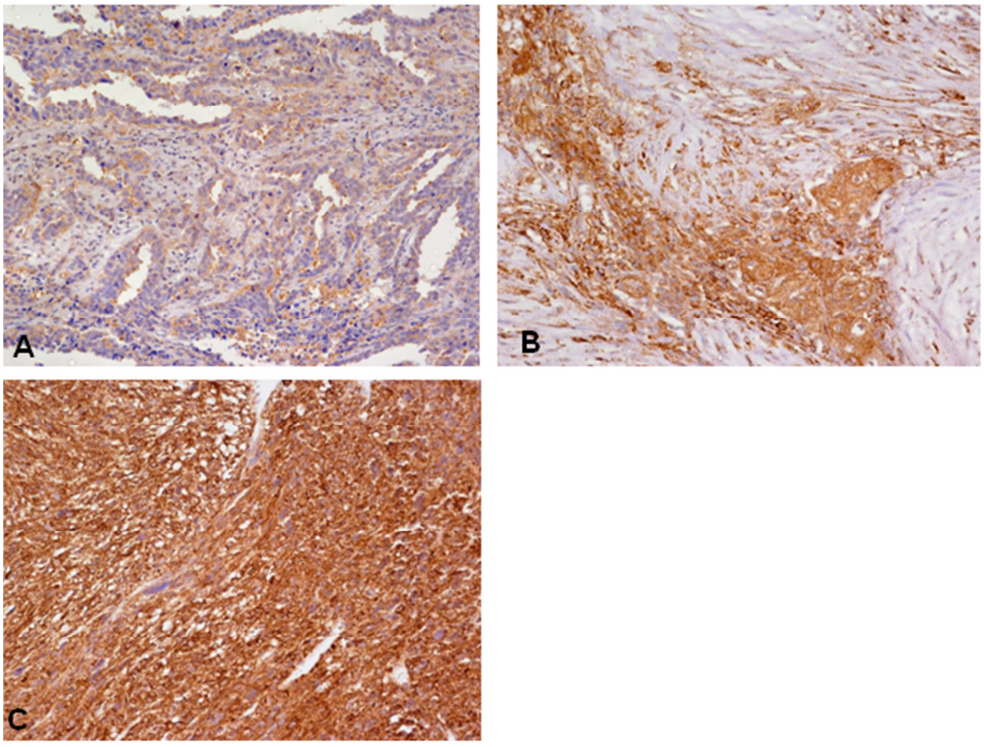


Figure 3: PD-L1 expression in endometrial biopsies. A. endometrial carcinoma biopsy showing weak expression (intensity score 1, 20x enlarged). B. endometrial carcinoma expressing moderate (intensity score 2) PD-L1 levels (20x enlarged). C. high PD-L1 expression levels in uterine sarcoma (20x enlarged). High expression can clearly be seen on the cell membrane (top of picture).

The number of analyzed samples and the corresponding percentages for each group are summarized in Table 3. When analyzing the expression level, we found an up-regulation in primary sarcomas in comparison with normal endometrium ($p < 0.01$). Overall, the expression level of PD-L1 was moderate to high. Of the patients with primary EMCAR, 72 % showed high PD-L1 expression, and for US, all patients were PD-L1^{hi} which shows that interference with the PD-1/PD-L1 axis could represent a promising new treatment option for uterine cancer patients. For EMCAR, we noticed a trend toward improved survival for patients with PD-L1^{hi} tumors ($p = 0.084$, data not shown).

Table 3: Immune checkpoint and IDO expression in different sample types.

Sample	**IDO**		**PD-L1**		**PD-L2**		**B7-H4**	
	N	**% positive**	**N**	**% positive**	**N**	**% positive**	**N**	**% positive**
nl EM	14	57%	16	81%	15	47%	15	100%
pr EMCAR	28	36%	29	83%	30	40%	30	100%
rec EMCAR	11	60%	9	67%	5	60%	11	100%
meta EMCAR	9	44%	9	100%	9	44%	10	90%
pr US	22	14%	21	100%	19	32%	26	100%
rec US	13	15%	11	100%	4	0%	13	92%
meta US	13	15%	10	100%	11	27%	13	100%

PD-L2 expression

The expression of PD-L2 was different from the expression of PD-L1 (Table 3). We found that 47% of normal endometria expressed PD-L2. In EMCAR in general, the percentage of positive tumor samples varied from 40 to 70%, while in the US group, this ranged from 0 to 32 % positive tumors. In general, the intensity scores for all the biopsies were low to moderate, and we did not find any differences in expression levels between any of the analyzed groups (Fig. 4B). Next to intracytoplasmic expression in tumor cells, we found the presence of PD-L2^{+} cells, possibly reflecting immune cell infiltrates in 10/30 (33 %) analyzed primary EMCAR biopsies as well as in 2/9 (22 %) of the included metastatic EMCAR lesions. The infiltrating cells were found intratumorally, in the tumoral stroma and in the tumor periphery (Fig. 5).

Overall in primary tumors, 30 % of EMCAR patients and 16 % of US patients showed high PD-L2 expression, thereby demonstrating the potential of PD-L2 blockade in a limited proportion of uterine cancer patients.

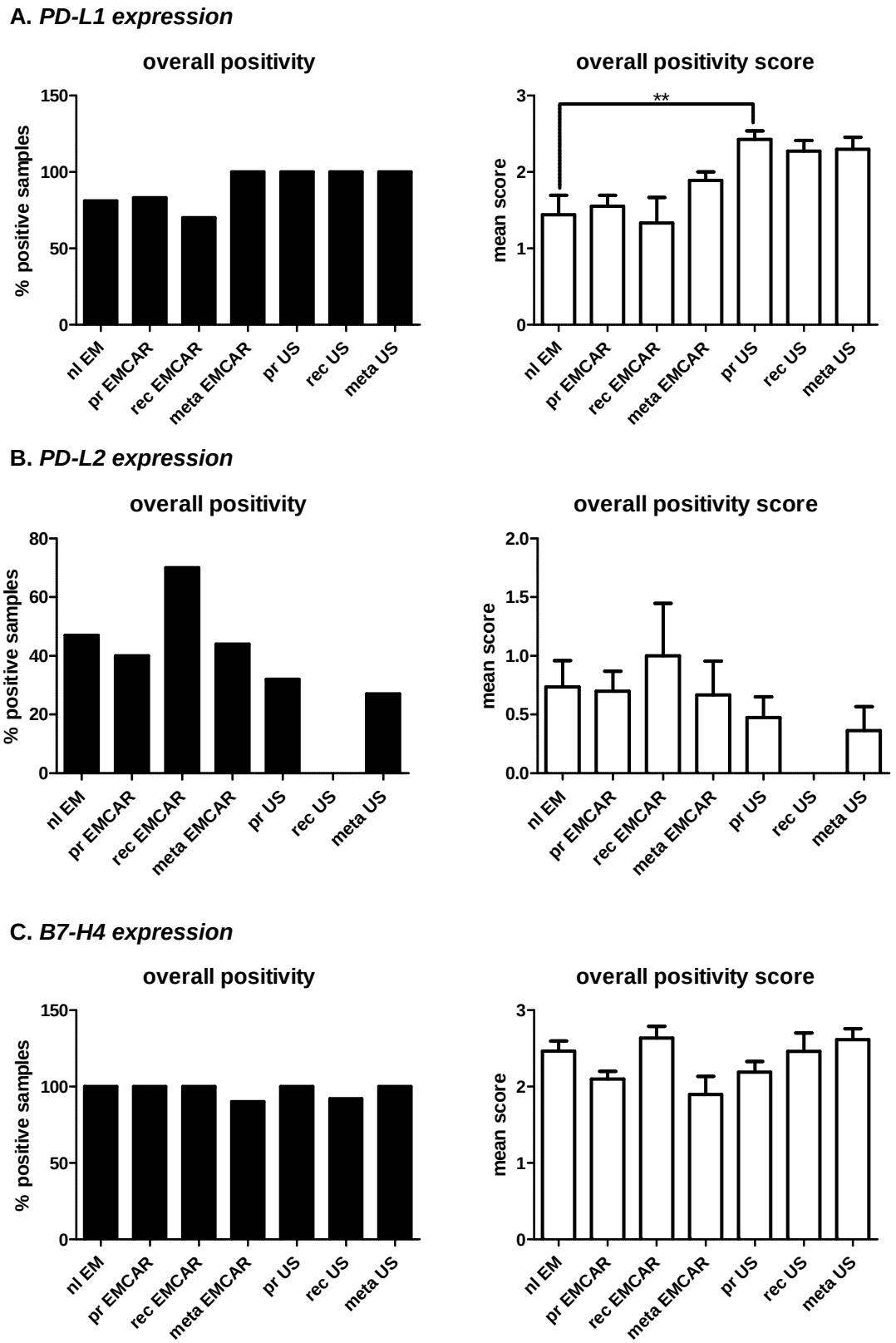


Figure 4: Immune checkpoints in uterine cancer. Left panels: percentage of positive tumors/normal endometrium. Right panels: Overall expression levels between groups. A. IHC analysis of PD-L1 expression. B. IHC analysis of PD-L2 expression. C. IHC analysis of B7-H4 expression. Statistical analysis was performed using the Kruskal-Wallis test in combination with the Dunn's Multiple Comparison Test.

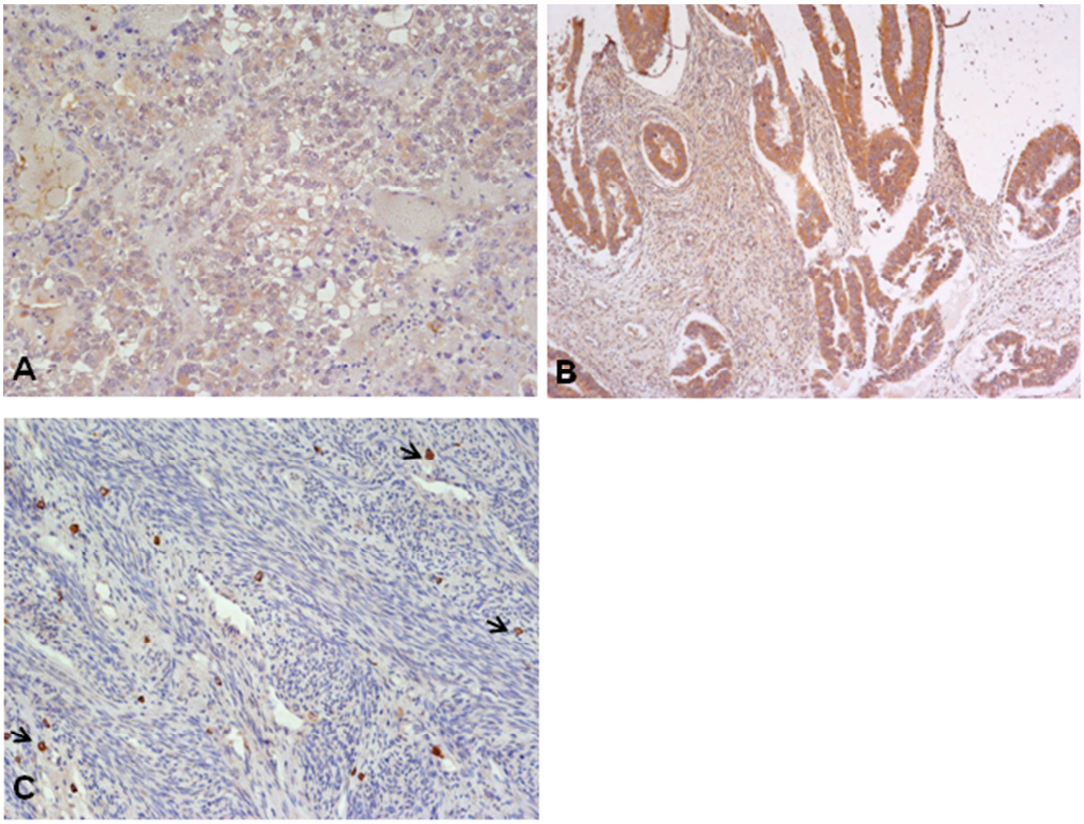


Figure 5: PD-L2 expression in endometrial biopsies. Endometrial carcinoma samples showing A. weak expression (20x enlarged) and B. moderate expression level (10x enlarged) of PD-L2. C. representative biopsy showing the presence of PD-L2 positive cells infiltrating the tumor periphery, possibly reflecting the presence of immune cells (20x enlarged).

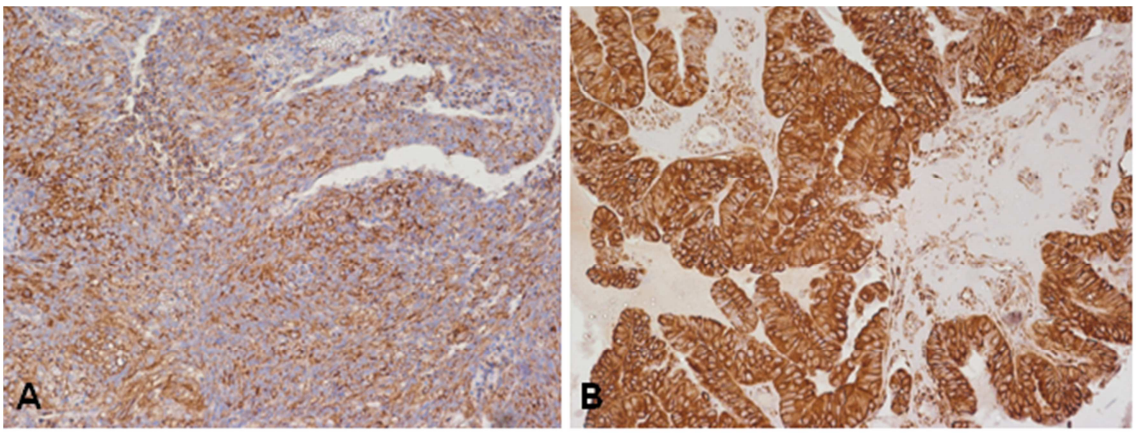


Figure 6: B7-H4 expression in endometrial biopsies. Endometrial carcinoma biopsies showing A. moderate B7-H4 expression (20x enlarged) and B. strong expression level (20x enlarged) with in addition very clear cytoplasmic and circumferential B7-H4 presence.

B7-H4 expression

B7-H4 is expressed in the vast majority of the currently analyzed biopsies (Table 3). In-depth analysis of the expression levels revealed no differences between any of the groups (Fig. 4C). B7-H4 is expressed exclusively in the cytoplasm(Fig. 6). Due to the visual aspects of the staining, discrimination of infiltrating cells was not possible.

The high expression levels of B7-H4 in both primary EMCAR (90 % of samples) and primary US (92 % of samples) make this an attractive candidate for uterine cancer therapy.

Galectin-1 and galectin-3 levels

For galectin-3, it was determined whether it is expressed on EMCAR and US cell lines (primary cell lines generated in our lab, kind gift from Dr. Coenegrachts) by flow cytometric analysis, and limited galectin-3 expression was detected on the plasma membrane, but nearly complete intracellular expression was found in these cell lines (Table 4).

Table 4: Galectin-3 expression in primary cancer cell lines**.**

Cell line	Histology	Grade	FIGO stage	% positive cells	
				gal-3-M[1]	gal-3-C[2]
PC-EM-002	Endometrioid	2	II	0.54	85
PC-EM-005	Endometrioid	3	II	3	98
PC-EM-012	Endometrioid	2	IV	8	60
PC-EM-018	Mixed	3	Ia	0.81	98
PC-EM-033	Endometrioid	3	Ia	1	99
PC-EM-046	Endometrioid	3	Ib	5	80
PC-EM-076	Endometrioid	2	Ia	1,2	99,8
PC-SAR-25	Embryonal rhabdomyosarcoma	NA	unknown	0,3	99,1

[1] gal-3-M: membranous detection, [2] gal-3-C: cytoplasmic detection

Galectin-1 and 3 presence in tumor tissue lysates was tested by ELISA in 29 EMCAR patients, 30 US patients, 21 normal/benign endometria and 18 normal myometria. The characteristics of the patients used for this study are summarized in Table 5. We found that galectin-1 is present in all of the different analyzed tissues, without significant differences between the entire sample groups (Fig. 7A). When subdividing the US group, a significant galectin-1 up-regulation was found in leiomyosarcomas compared to myometrium ($p < 0.01$), carcinosarcomas ($p < 0.05$) and other US subtypes ($p < 0.05$). For galectin-3 we found a significantly higher intratumoral level in EMCAR samples compared with US samples ($p < 0.05$), shown in Fig. 7B.

To further assess which specific cell type of the tumor expresses galectins, their expression on fibroblasts, immune cells and tumor cells was determined in single-cell suspensions from fresh tumor biopsies (tumor characteristics shown in Table 6). Considerable variation in the cellular composition of the single-cell tumor suspensions: 1.24 – 16.16% fibroblasts, 15.19 – 75.07% immune cells and 6.73 – 80.21% tumor cells (data not shown) was found. When gating on these different cell

populations, a slightly higher percentage of membranous galectin-3 positivity on tumor cells versus fibroblasts and immune cells was found, although this did not reach statistical significance, shown in Fig. 7C. With regard to intracellular galectin-3 expression, the majority of fibroblasts, immune cells and tumor cells expressed galectin-3. Intracellular galectin-3 expression was significantly higher in tumor cells versus fibroblasts ($p < 0.05$).

Table 5: Clinical characteristics of tissue lysates used for galectin detection.

Patient characteristics	EMCAR patients	US patients
Total	29	30
Age (years)		
Mean	67.1	61
Range	48-86	29-83
FIGO stage (2009)		
I	9 (31.0%)	
II	8 (27.6%)	
III	8 (27.6%)	
IV	4 (13.8%)	
Differentiation grade (primary tumor)		
Low	10 (34.5%)	
Moderate	6 (20.7%)	
High	13 (44.8%)	
Histologic subtype (primary tumor)		
Endometrioid	22 (75.9%)	
Clear cell	3 (10,35%)	
Serous papillary	3 (10.35%)	
Mixed	1 (3.4%)	
Carcinosarcoma		10 (33.3%)
Leiomyosarcoma		8 (26.6%)
Rhabdomyosarcoma		3 (10%)
Endometrial stromal sarcoma		7 (23.3%)
Unknown		1 (3.3%)
Ewing sarcoma		1 (3.3%)

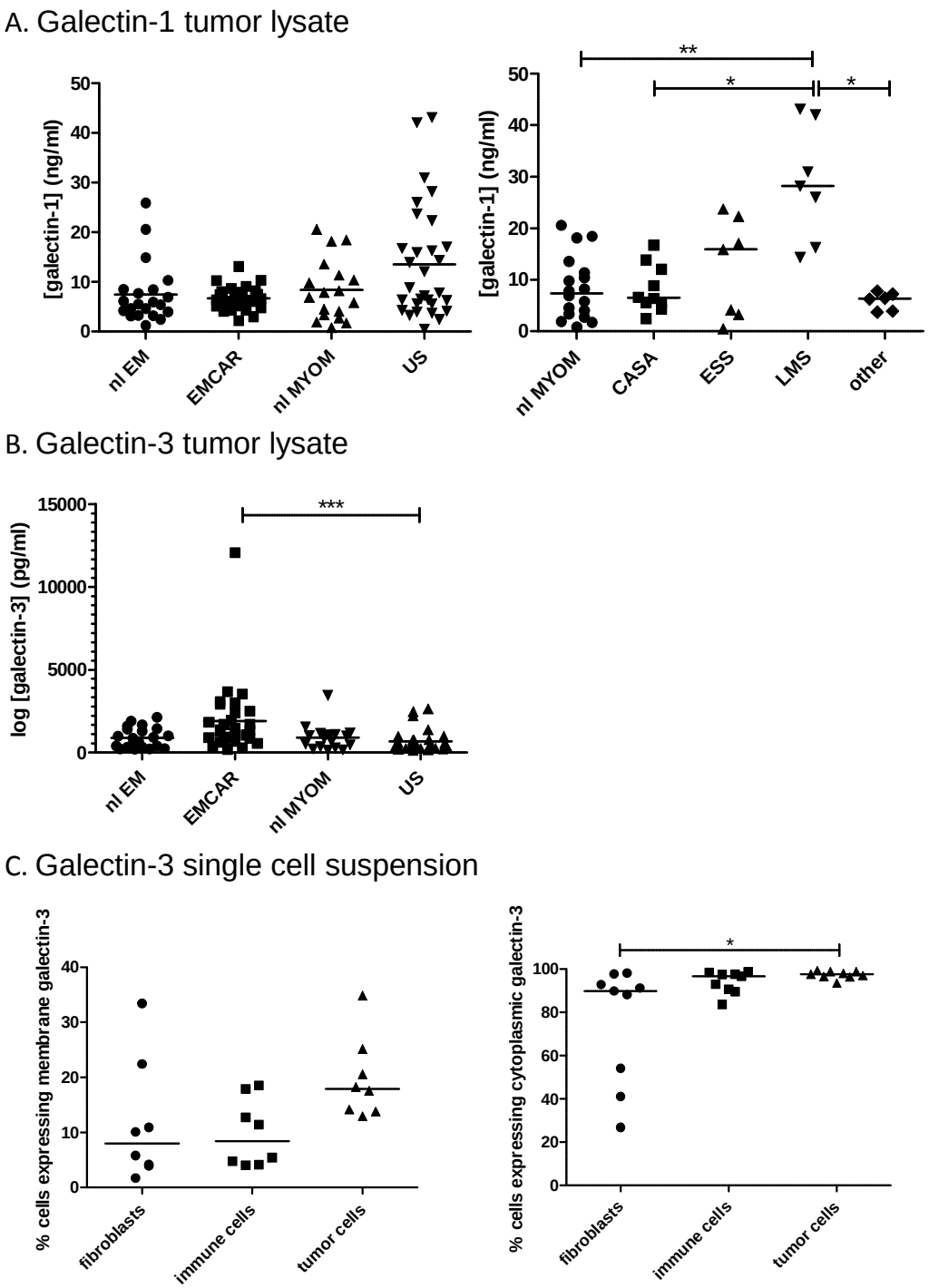


Figure 7: Galectin expression in patient plasma and uterine tissue samples. A. galectin-1 expression in uterine tissue. Left panel: overall galectin-1 expression. Right panel: galectin-1 expression in myometrium versus sarcoma histological subtypes. B. Galectin-3 expression in uterine tissue. C. Flow cytometric detection of galectin-3 on different cell populations within single-cell tumor suspensions. Left panel: membranous galectin-3 expression. Right panel intracellular galectin-3 expression. Groups were analyzed statistically by one-way ANOVA followed by Tukey's Multiple Comparison Test or the by Kruskal-Wallis test followed by the Dunn's Multiple Comparison Test, depending on Gaussian distribution.

Table 6: Characteristics of tumors used for flow cytometric assessment of galectin-3

ID	Type	FIGO stage	Histology	Grade	Time point
13	EMCAR	IIIc	serous	3	recurrence
17	US	IV	leiomyosarcoma	3	primary
18	EMCAR	Ia	endometrioid	1	primary
19	EMCAR	Ia	endometrioid	1	primary
22	US	Ib	leiomyosarcoma	3	recurrence
24	EMCAR	Ia	endometrioid	2	primary
25	EMCAR	II	endometrioid	1/2	primary
26	EMCAR	Ia	endometrioid	1	primary
27	EMCAR	Ib	endometrioid	3	primary

Arginase-1 activity and MDSC infiltration

Arginase-1 activity was measured in tissue lysates of 25 normal endometria, 39 EMCAR, 27 US and 18 normal myometria. Clinical parameters of all patient samples are depicted in Table 7. Activity was significantly up-regulated in EMCAR samples compared with normal endometria ($p < 0.0001$). No difference in activity was found between normal myometria and US samples (Fig 8A). These data suggested a possible implication of MDSC in endometrial carcinoma and inspired us to explore whether MDSC infiltration can be found in EMCAR and US tumors. Therefore, single-cell suspensions from fresh tumor biopsies were prepared (tumor characteristics shown in Supplemental Table 6), and flow cytometric analysis to detect MDSC and assess their arginase-1 expression was performed. MDSC infiltration was indeed detected in tumor specimens, and these cells were mainly of granulocytic phenotype (lin^- HLA-$DR^{lo/-}$ $CD11b^+$ $CD14^-$; Fig 8B). Tumors contained significantly more granulocytic compared with monocytic MDSC ($p=0.0124$). However, both MDSC subtypes expressed similar arginase-1 levels (Fig. 8C). The majority of the granulocytic MDSC were $CD15^+$, with approximately half of them co-expressing CD33. The monocytic MDSC were mainly $CD15^+$ $CD33^+$ (approximately 60 %), with an additional 24 % of $CD15^+$ $CD33^-$ cells (data not shown).

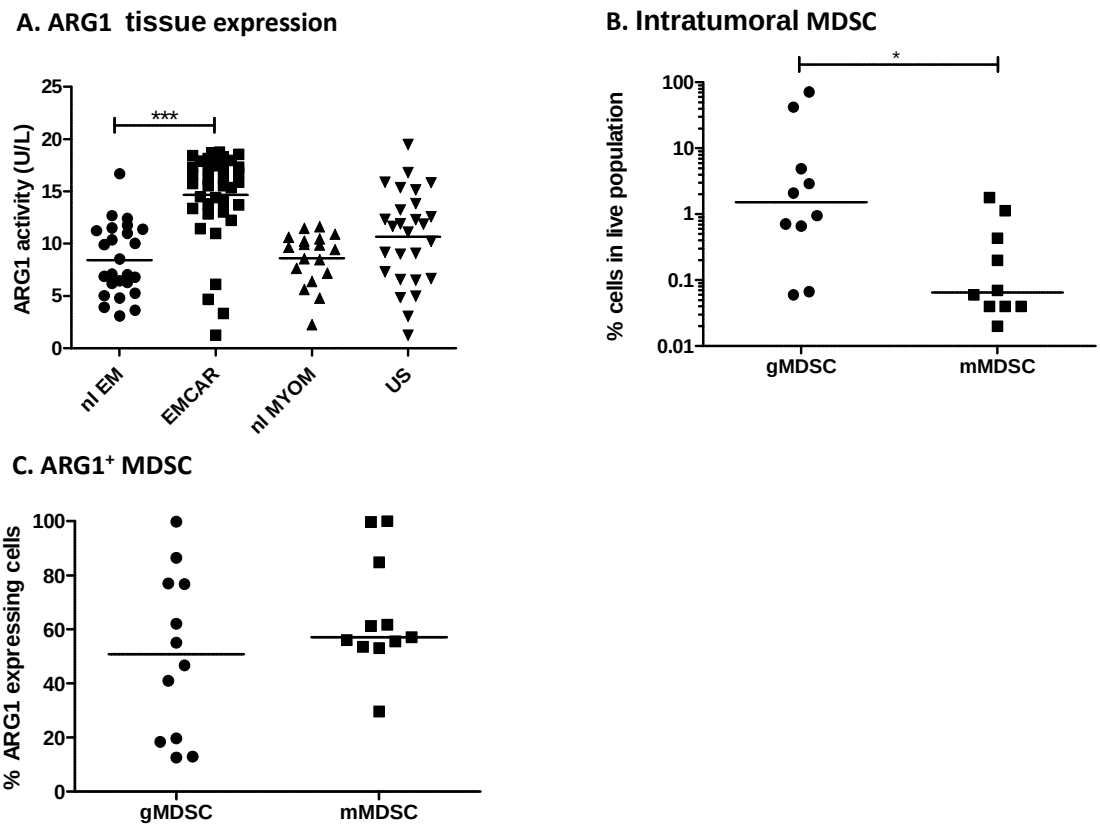


Figure 8: Arginase-1 activity in uterine tissues and MDSC infiltration in uterine tumors. A. Arginase-1 activity in uterine tissues. B. MDSC infiltration in uterine tumors. C. Arginase-1 expression by MDSC infiltrating uterine tumors. Data were analyzed statistically using the unpaired t-test or the Mann Whitney test (nl-EM vs EMCAR or nl MYOM vs US in A), depending on Gaussian distribution.

Table 7: Clinical characteristics of tissue lysates used for arginase-1 activity detection.

Characteristics	EMCAR patients	US patients
Total	39	27
Age (years)		
Mean	67.2	61
Range	36-86	29-83
FIGO stage		
I	10 (25.64%)	
II	10 (25.64%)	
III	9 (23.08%)	
IV	10 (25.64%)	
Differentiation grade		
Low	8 (20.51%)	
Moderate	4 (10.26%)	
High	17 (43.59%)	
Unknown	10 (25.64%)	
Histologic subtype (primary tumor)		
Endometrioid	20 (51.3%)	
Clear cell	8 (12.8%)	
Serous papillary	11 (28.2%)	
Mixed	3 (7.7%)	
Carcinosarcoma		9 (33.3%)
Leiomyosarcoma		8 (29.6%)
Rhabdomyosarcoma		2 (7.4%)
Endometrial stromal sarcoma		6 (22.2%)
Unknown		1 (3.75%)
Ewing sarcoma		1 (3.75%)

Table 8: Characteristics of tumors used for MDSC infiltration and arginase-1 expression by MDSC.

ID	Type	FIGO stage	Histology	Grade	Time point	MDSC frequency	MDSC arginase-1
5	EMCAR	Ia	endometrioid	1	primary	yes	no
9	EMCAR	IIIa	mixed serous-clear cell	3	primary	yes	no
10	EMCAR	Ib	endometrioid	1	primary	yes	yes
11	EMCAR	Ia	endometrioid	1	primary	yes	yes
14	EMCAR	Ib-II	endometrioid	3	primary	yes	yes
17	US	IV	leiomyosarcoma	3	primary	no	yes
18	EMCAR	Ia	endometrioid	1	primary	no	yes
19	EMCAR	Ia	endometrioid	1	primary	no	yes
22	US	Ib	leiomyosarcoma	3	recurrence	no	yes
24	EMCAR	Ia	endometrioid	2	primary	yes	yes
25	EMCAR	II	endometrioid	1/2	primary	yes	yes
26	EMCAR	Ia	endometrioid	1	primary	yes	yes
27	EMCAR	Ib	endometrioid	3	primary	yes	yes
28	US	IV	myxoid		primary	yes	yes

DISCUSSION

In the current research, we have evaluated the expression of PD-L1, PD-L2, B7-H4, IDO, galectin-1 and galectin-3 in uterine tumors and documented the arginase-1 activity and MDSC infiltration in these tumors. To our knowledge, this is the first paper to describe the presence of PD-L1 and PD-L2 in endometrial cancer as such, and we are not aware that any of the currently described molecules have been described in uterine sarcoma so far, except for possibly IDO [31], galectin-1 [32] and galectin-3 [32-34].

Both PD-1 ligands have previously been implicated in different types of cancer, although more is known about PD-L1 expression in several types of tumors, such as melanoma, brain tumors, lung cancer, urothelial cancer and pancreatic cancer [7, 35-38]. In soft tissue sarcomas in general, one study found expression of PD-L1 in 65 % of cases [9]. To date, little information is available on the expression of both ligands in gynecological tumors in general. Our data on PD-L1 expression show that it is expressed in the vast majority of both normal endometrial samples and of endometrial tumors, with no difference in proportion or expression level between any of the analyzed groups. In contrast to what we anticipated, we observed a trend toward improved overall survival in patients with PD-L1^{hi} tumors. However, this phenomenon has previously been described in melanoma, merkel cell carcinoma and MMR-proficient colorectal carcinoma [39-41]. Up-regulation of PD-L1 has been shown to be driven by IFNγ and CD8^{+} T cells. Hence, PD-L1 expression could be viewed as a result of an ongoing endogenous anti-tumor immune response and thus may represent a negative feedback loop dependent on an infiltrating immune response [42, 43].

PD-L2 has long been thought to be present only on dendritic cells and macrophages. However, more evidence is beginning to emerge of PD-L2 presence on somatic tissues and in cancer as well. It has, for example, been shown to be present in cancer-associated fibroblasts (Nazareth et al. in [10]) as well as in esophageal [13] and hepatocellular carcinoma [11]. Most of the currently analyzed biopsies

were negative for PD-L2, except in the recurrent carcinoma group. Similar to our results of low expression in the majority of tumors, a study in ovarian cancer has shown that the majority of the tumors are negative for PD-L2 [12]. Both ligands have also been investigated in cervical cancer [44], both being present in only a minority of the investigated samples.

B7-H4 has been shown in different tumors, summarized by He et al. [14]. Quite some data are available on its presence in breast cancer and ovarian cancer. It has been shown to be present intracellularly in ovarian tumor cells and on tumor-associated macrophages (TAM) in these tumors [45, 46]. B7-H4 expression levels were correlated with patient outcome [45]. Two studies report on B7-H4 expression in endometrial cancer [47, 48]. Both studies show B7-H4 expression in normal endometrium, and B7-H4 staining was shown to increase from normal to malignant endometrial glands. Our results are somewhat contradictory since we found high B7-H4 levels in almost all of the analyzed tissue samples in both benign and malignant endometrial tissues as well as in US samples. This observation might be due to the use of different antibodies, staining protocols and/or scoring protocols.

The immune inhibitory enzyme IDO has also been shown to play an important role in cancer-induced immunosuppression by depriving T cells of tryptophan and thus preventing their activation [49]. Uyttenhove et al. described IDO in 25 different tumors [31]. IDO has previously been detected in ovarian [31, 50] as well as endometrial tumors [29-31, 51, 52]. We have found high IDO expression in 21 % of primary endometrial tumors, which is in line with data reported by De Jong et al. (18,1 %), but lower than the results obtained by Ino et al. (46-49 %). We have also found an increased IDO expression level in recurrent EMCAR compared with primary EMCAR, pointing to increased immunosuppression in recurrent tumors. We found IDO expression only in a minority of US, in corroboration with Uyttenhove et al., who found IDO expression in 20% of sarcomas. However, the exact subtype of sarcoma is not specified any further [31]. Except for PD-L1, no correlation between high and low expression and patient overall survival was found (data not shown). However, the currently analyzed sample population is too small to perform sound survival analyses. Based on the current data, no definitive conclusion can be made regarding patient survival. When analyzing the current sample population, no differences were found regarding FIGO stage, histological subtype or histological grade, except for a significantly lower expression level of IDO in grade 3 tumors compared to grade 1 ($p < 0.05$; data not shown). In addition, we could not find a difference in expression level between type I and type II EMCAR for any of the analyzed mediators. We do, however, acknowledge that the current sample sizes are too small to perform adequate statistical analyses regarding sub-parameters, which may bias or mask possible differences in expression levels.

Galectin-1 and galectin-3 have both been described in tumors. Galectin-1 and galectin-3 are both up-regulated in among others bladder carcinoma [53, 54], head and neck squamous cell carcinoma (HNSCC) [55] and ovarian cancer [56, 57]. Several studies have been published on galectins in endometrial cancer, with contradictory results. Galectin-1, but not galectin-3, has been shown to be increased in EMCAR compared with normal endometrium [58]. Similarly, galectin-1 was found to be increased in endometrioid EMCAR from well differentiated to undifferentiated carcinoma [59]. Contradictory to the data by Van den Brule et al. [58], Brustmann et al. were able to show an increase in galectin-3 in EMCAR samples compared with normal endometrium samples [60]. However, Ege et al. reported decreased galectin-3 expression in EMCAR samples compared with normal endometrium [61]. We currently found no increased galectin-1 or galectin-3 expression in

tissue lysates of EMCAR or US compared with normal/benign controls. However, galectin-1 levels in lysates of leiomyosarcomas were significantly higher compared with myometrium, carcinosarcomas and other histological subtypes of US. We further showed both membranous and cytoplasmic expression of galectin-3 on single-cell tumor suspensions from primary tumors and on uterine cancer cell lines. In cancer, there is often a change in intracellular localization of galectin-3. Brustmann et al. found an increased nuclear expression in EMCAR [60]. Data of Mylonas et al. showed that it can be detected in both the cytoplasm and the nucleus [58], but exclusively cytoplasmic expression was associated with increased myometrial invasion of EMCAR [58]. In the current study, we used tissue lysates or membrane/intracellular flow cytometric analysis, and consequently, no analysis on galectin location could be done to corroborate these data. Weissenbacher et al. analyzed the expression of galectin-1 and galectin-3 in uterine leiomyosarcoma. Contradictory to what we currently found in the total sarcoma population for galectin-1, they found no difference in galectin-1 or galectin-3 expression when compared with normal myometrium [34]. This discrepancy may in part be explained by the use of a semi-quantitative versus a quantitative analysis method in the current setting, which creates some difficulties for precise comparison of results.

Myeloid-derived suppressor cells have been shown to play an important role in cancer immunosuppression. de Boniface et al. showed increased expression of arginase-1 on MDSC in breast cancer patients, which correlated with tumor grade [62]. Arginase-releasing MDSC exerting detrimental effects on T cells are also increased in patient blood of renal cell carcinoma (RCC) patients [63, 64]. We have currently analyzed the activity of arginase-1 as a surrogate for MDSC. We found increased arginase-1 activity in EMCAR lysates compared with normal endometrium, indicating a possible involvement of MDSC in EMCAR. Furthermore, we showed for the first time that both EMCAR and US tumors contain MDSC infiltrates that express arginase-1.

In conclusion, we report the presence of the immune checkpoints PD-L1/PD-L2 and B7-H4 in uterine tumors as well as the presence of the immune inhibitory molecules IDO, arginase-1, galectin-1 and galectin-3 and the immunosuppressive MDSC. The current data in conjunction with positive results of currently ongoing clinical trials and preclinical studies with inhibitors for these molecules advocate for a possible future use in patients suffering from uterine tumors. In our opinion, IDO and PD-L2 are useful targets for immune inhibition in a minority of uterine cancer patients. Both PD-L1 and B7-H4 could represent targets in both tumor types, considering their high expression levels. Finally, intervention with MDSC function offers a new treatment perspective in EMCAR patients.

REFERENCES

1. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12(4):252-64.
2. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med. 2012;366(26):2455-65.
3. Topalian SL, Drake CG, Pardoll DM. Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. Curr Opin Immunol. 2012;24(2):207-12.
4. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med. 2012;366(26):2443-54.
5. Blank C, Gajewski TF, Mackensen A. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. Cancer Immunol Immunother. 2005;54(4):307-14.

6. Fife BT, Bluestone JA. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. Immunol Rev. 2008;224:166-82.
7. Hino R, Kabashima K, Kato Y, Yagi H, Nakamura M, Honjo T, et al. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. Cancer. 2010;116(7):1757-66.
8. Mu CY, Huang JA, Chen Y, Chen C, Zhang XG. High expression of PD-L1 in lung cancer may contribute to poor prognosis and tumor cells immune escape through suppressing tumor infiltrating dendritic cells maturation. Med Oncol. 2011;28(3):682-8.
9. Kim JR, Moon YJ, Kwon KS, Bae JS, Wagle S, Kim KM, et al. Tumor Infiltrating PD1-Positive Lymphocytes and the Expression of PD-L1 Predict Poor Prognosis of Soft Tissue Sarcomas. PloS one. 2013;8(12):e82870.
10. Rozali EN, Hato SV, Robinson BW, Lake RA, Lesterhuis WJ. Programmed death ligand 2 in cancer-induced immune suppression. Clin Dev Immunol. 2012;2012:656340.
11. Gao Q, Wang XY, Qiu SJ, Yamato I, Sho M, Nakajima Y, et al. Overexpression of PD-L1 significantly associates with tumor aggressiveness and postoperative recurrence in human hepatocellular carcinoma. Clin Cancer Res. 2009;15(3):971-9.
12. Hamanishi J, Mandai M, Iwasaki M, Okazaki T, Tanaka Y, Yamaguchi K, et al. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. Proc Natl Acad Sci U S A. 2007;104(9):3360-5.
13. Ohigashi Y, Sho M, Yamada Y, Tsurui Y, Hamada K, Ikeda N, et al. Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. Clin Cancer Res. 2005;11(8):2947-53.
14. He C, Qiao H, Jiang H, Sun X. The inhibitory role of b7-h4 in antitumor immunity: association with cancer progression and survival. Clin Dev Immunol. 2011;2011:695834.
15. Oikonomopoulou K, Li L, Zheng Y, Simon I, Wolfert RL, Valik D, et al. Prediction of ovarian cancer prognosis and response to chemotherapy by a serum-based multiparametric biomarker panel. Br J Cancer. 2008;99(7):1103-13.
16. Simon I, Katsaros D, Rigault de la Longrais I, Massobrio M, Scorilas A, Kim NW, et al. B7-H4 is over-expressed in early-stage ovarian cancer and is independent of CA125 expression. Gynecol Oncol. 2007;106(2):334-41.
17. Yi KH, Chen L. Fine tuning the immune response through B7-H3 and B7-H4. Immunol Rev. 2009;229(1):145-51.
18. Munn DH. Blocking IDO activity to enhance anti-tumor immunity. Front Biosci (Elite Ed). 2012;4:734-45.
19. Munn DH, Mellor AL. Indoleamine 2,3 dioxygenase and metabolic control of immune responses. Trends Immunol. 2013;34(3):137-43.
20. Yang RY, Rabinovich GA, Liu FT. Galectins: structure, function and therapeutic potential. Expert Rev Mol Med. 2008;10:e17.
21. Demotte N, Wieers G, Van Der Smissen P, Moser M, Schmidt C, Thielemans K, et al. A galectin-3 ligand corrects the impaired function of human CD4 and CD8 tumor-infiltrating lymphocytes and favors tumor rejection in mice. Cancer Res. 2010;70(19):7476-88.
22. Munder M. Arginase: an emerging key player in the mammalian immune system. Br J Pharmacol. 2009;158(3):638-51.
23. Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res. 2004;64(16):5839-49.
24. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. Blood. 2007;109(4):1568-73.
25. Nagaraj S, Gabrilovich DI. Myeloid-derived suppressor cells in human cancer. Cancer J. 2010;16(4):348-53.
26. Huang A, Zhang B, Wang B, Zhang F, Fan KX, Guo YJ. Increased CD14(+)HLA-DR (-/low) myeloid-derived suppressor cells correlate with extrathoracic metastasis and poor response to

chemotherapy in non-small cell lung cancer patients. Cancer Immunol Immunother. 2013;62(9):1439-51.
27. Meyer C, Cagnon L, Costa-Nunes CM, Baumgaertner P, Montandon N, Leyvraz L, et al. Frequencies of circulating MDSC correlate with clinical outcome of melanoma patients treated with ipilimumab. Cancer Immunol Immunother. 2013.
28. Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C, et al. Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nat Med. 2012;18(8):1254-61.
29. Ino K, Yoshida N, Kajiyama H, Shibata K, Yamamoto E, Kidokoro K, et al. Indoleamine 2,3-dioxygenase is a novel prognostic indicator for endometrial cancer. Br J Cancer. 2006;95(11):1555-61.
30. de Jong RA, Kema IP, Boerma A, Boezen HM, der Want JJ, Gooden MJ, et al. Prognostic role of indoleamine 2,3-dioxygenase in endometrial carcinoma. Gynecol Oncol. 2012;126(3):474-80.
31. Uyttenhove C, Pilotte L, Theate I, Stroobant V, Colau D, Parmentier N, et al. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med. 2003;9(10):1269-74.
32. Schwarz G, Jr., Remmelink M, Decaestecker C, Gielen I, Budel V, Burchert M, et al. Galectin fingerprinting in tumor diagnosis. Differential expression of galectin-3 and galectin-3 binding sites, but not galectin-1, in benign vs malignant uterine smooth muscle tumors. Am J Clin Pathol. 1999;111(5):623-31.
33. Weissenbacher T, Vrekoussis T, Roeder D, Makrigiannakis A, Mayr D, Ditsch N, et al. Analysis of Epithelial Growth Factor-Receptor (EGFR) Phosphorylation in Uterine Smooth Muscle Tumors: Correlation to Mucin-1 and Galectin-3 Expression. Int J Mol Sci. 2013;14(3):4783-92.
34. Weissenbacher T, Kuhn C, Mayr D, Pavlik R, Friese K, Scholz C, et al. Expression of mucin-1, galectin-1 and galectin-3 in human leiomyosarcoma in comparison to leiomyoma and myometrium. Anticancer Res. 2011;31(2):451-7.
35. Inman BA, Sebo TJ, Frigola X, Dong H, Bergstralh EJ, Frank I, et al. PD-L1 (B7-H1) expression by urothelial carcinoma of the bladder and BCG-induced granulomata: associations with localized stage progression. Cancer. 2007;109(8):1499-505.
36. Jacobs JF, Idema AJ, Bol KF, Nierkens S, Grauer OM, Wesseling P, et al. Regulatory T cells and the PD-L1/PD-1 pathway mediate immune suppression in malignant human brain tumors. Neuro Oncol. 2009;11(4):394-402.
37. Konishi J, Yamazaki K, Azuma M, Kinoshita I, Dosaka-Akita H, Nishimura M. B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. Clin Cancer Res. 2004;10(15):5094-100.
38. Zhang Y, Huang S, Gong D, Qin Y, Shen Q. Programmed death-1 upregulation is correlated with dysfunction of tumor-infiltrating CD8+ T lymphocytes in human non-small cell lung cancer. Cell Mol Immunol. 2010;7(5):389-95.
39. Taube JM, Anders RA, Young GD, Xu HY, Sharma R, McMiller TL, et al. Colocalization of Inflammatory Response with B7-H1 Expression in Human Melanocytic Lesions Supports an Adaptive Resistance Mechanism of Immune Escape. Sci Transl Med. 2012;4(127).
40. Droeser RA, Hirt C, Viehl CT, Frey DM, Nebiker C, Huber X, et al. Clinical impact of programmed cell death ligand 1 expression in colorectal cancer. Eur J Cancer. 2013;49(9):2233-42.
41. Lipson EJ, Vincent JG, Loyo M, Kagohara LT, Luber BS, Wang H, et al. PD-L1 expression in the merkel cell carcinoma microenvironment: association with inflammation, merkel cell polyomavirus, and overall survival. Cancer Immunol Res. 2013;1(1):54-63.
42. Spranger S, Spaapen RM, Zha YY, Williams J, Meng YR, Ha TT, et al. Up-Regulation of PD-L1, IDO, and T-regs in the Melanoma Tumor Microenvironment Is Driven by CD8(+) T Cells. Sci Transl Med. 2013;5(200).
43. Lyford-Pike S, Peng SW, Young GD, Taube JM, Westra WH, Akpeng B, et al. Evidence for a Role of the PD-1:PD-L1 Pathway in Immune Resistance of HPV-Associated Head and Neck Squamous Cell Carcinoma. Cancer Res. 2013;73(6):1733-41.

44. Karim R, Jordanova ES, Piersma SJ, Kenter GG, Chen L, Boer JM, et al. Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma. Clin Cancer Res. 2009;15(20):6341-7.
45. Kryczek I, Wei S, Zhu G, Myers L, Mottram P, Cheng P, et al. Relationship between B7-H4, regulatory T cells, and patient outcome in human ovarian carcinoma. Cancer Res. 2007;67(18):8900-5.
46. Kryczek I, Zou L, Rodriguez P, Zhu G, Wei S, Mottram P, et al. B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. J Exp Med. 2006;203(4):871-81.
47. Miyatake T, Tringler B, Liu W, Liu SH, Papkoff J, Enomoto T, et al. B7-H4 (DD-O110) is overexpressed in high risk uterine endometrioid adenocarcinomas and inversely correlated with tumor T-cell infiltration. Gynecol Oncol. 2007;106(1):119-27.
48. Qian Y, Shen L, Cheng LF, Wu ZG, Yao HP. B7-H4 expression in various tumors determined using a novel developed monoclonal antibody. Clin Exp Med. 2011;11(3):163-70.
49. Prendergast GC. Immune escape as a fundamental trait of cancer: focus on IDO. Oncogene. 2008;27(28):3889-900.
50. Okamoto A, Nikaido T, Ochiai K, Takakura S, Saito M, Aoki Y, et al. Indoleamine 2,3-dioxygenase serves as a marker of poor prognosis in gene expression profiles of serous ovarian cancer cells. Clin Cancer Res. 2005;11(16):6030-9.
51. Ino K, Yamamoto E, Shibata K, Kajiyama H, Yoshida N, Terauchi M, et al. Inverse correlation between tumoral indoleamine 2,3-dioxygenase expression and tumor-infiltrating lymphocytes in endometrial cancer: its association with disease progression and survival. Clin Cancer Res. 2008;14(8):2310-7.
52. Sedlmayr P, Semlitsch M, Gebru G, Karpf E, Reich O, Tang T, et al. Expression of indoleamine 2,3-dioxygenase in carcinoma of human endometrium and uterine cervix. Adv Exp Med Biol. 2003;527:91-5.
53. Cindolo L, Benvenuto G, Salvatore P, Pero R, Salvatore G, Mirone V, et al. galectin-1 and galectin-3 expression in human bladder transitional-cell carcinomas. Int J Cancer. 1999;84(1):39-43.
54. Demydenko D, Berest I. Expression of galectin-1 in malignant tumors. Exp Oncol. 2009;31(2):74-9.
55. Saussez S, Lorfevre F, Lequeux T, Laurent G, Chantrain G, Vertongen F, et al. The determination of the levels of circulating galectin-1 and -3 in HNSCC patients could be used to monitor tumor progression and/or responses to therapy. Oral Oncol. 2008;44(1):86-93.
56. Kim MK, Sung CO, Do IG, Jeon HK, Song TJ, Park HS, et al. Overexpression of Galectin-3 and its clinical significance in ovarian carcinoma. Int J Clin Oncol. 2011;16(4):352-8.
57. van den Brule F, Califice S, Garnier F, Fernandez PL, Berchuck A, Castronovo V. Galectin-1 accumulation in the ovary carcinoma peritumoral stroma is induced by ovary carcinoma cells and affects both cancer cell proliferation and adhesion to laminin-1 and fibronectin. Lab Invest. 2003;83(3):377-86.
58. van den Brule FA, Buicu C, Berchuck A, Bast RC, Deprez M, Liu FT, et al. Expression of the 67-kD laminin receptor, galectin-1, and galectin-3 in advanced human uterine adenocarcinoma. Hum Pathol. 1996;27(11):1185-91.
59. Mylonas I, Mayr D, Walzel H, Shabani N, Dian D, Kuhn C, et al. Mucin 1, Thomsen-Friedenreich expression and galectin-1 binding in endometrioid adenocarcinoma: an immunohistochemical analysis. Anticancer Res. 2007;27(4A):1975-80.
60. Brustmann H, Riss D, Naude S. Galectin-3 expression in normal, hyperplastic, and neoplastic endometrial tissues. Pathol Res Pract. 2003;199(3):151-8.
61. Ege CB, Akbulut M, Zekioglu O, Ozdemir N. Investigation of galectin-3 and heparanase in endometrioid and serous carcinomas of the endometrium and correlation with known predictors of survival. Arch Gynecol Obstet. 2011;284(5):1231-9.

62. de Boniface J, Mao Y, Schmidt-Mende J, Kiessling R, Poschke I. Expression patterns of the immunomodulatory enzyme arginase 1 in blood, lymph nodes and tumor tissue of early-stage breast cancer patients. Oncoimmunology. 2012;1(8):1305-12.
63. Rodriguez PC, Ernstoff MS, Hernandez C, Atkins M, Zabaleta J, Sierra R, et al. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. Cancer Res. 2009;69(4):1553-60.
64. Zea AH, Rodriguez PC, Atkins MB, Hernandez C, Signoretti S, Zabaleta J, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. Cancer Res. 2005;65(8):3044-8.

Part V clinical trials

Part V

Chapter 6 Immune monitoring of WT1 DC vaccination in uterine cancer patients

Coosemans A, **Vanderstraeten A,** Tuyaerts S, Verschuere T, Moerman P, Berneman Z, Vergote I, Amant F, Van Gool SW

Summarized and adapted from Coosemans et al. Anticancer Res. 2013 Dec;33(12):5495-500
Coosemans et al. Anticancer Res. 2013 Sep;33(9):3855-9

Immune monitoring of WT1 DC vaccination in uterine and ovarian cancer patients

ABSTRACT

Ovarian cancer and endometrial cancer are the two most common gynecological malignancies. Ovarian cancer often presents in late stages as an aggressive tumor and tumors often recur multiple times. Due to its aggressive disease course and the development of resistance to current treatment regimens, additional treatment options are necessary.
Endometrial cancer on the other hand, is mostly diagnosed in early stages and currently available treatment options for endometrial cancer are mostly curative. However, as is the case for ovarian cancer, treatment options are limited for far-advanced and recurrent endometrial tumors. Dendritic cell-based immunotherapy is a valid treatment option for both tumors.
Eight patients suffering from recurrent ovarian or uterine tumors were vaccinated with WT1 mRNA loaded DC and monitored for WT1-specific immune responses and clinical response. No severe adverse events or toxicities were reported, further confirming the feasibility and safety of DC vaccination. Post-vaccination immune responses could be detected in 5/8 patients. Transient clinical responses were seen in 2/8 patients.
Collectively, the currently obtained results indicate that WT1 DC vaccination in uterine cancer patients results in detectable immune responses with, for the current patients population, minimal clinical response.

INTRODUCTION

WT1 has been ranked as the most important tumor-associated antigen [1], making it an attractive target for immunotherapy. Since 2004, clinical case reports and studies have been published about WT1 immunotherapy. Most commonly, a modified WT1 peptide is injected with the goal of eliciting a WT1-specific T-cell response. Vaccination with autologous dendritic cells (DCs) loaded with WT1 peptides is another means to establish such a response. To date, 78 cases of solid tumors, including 8 uterine tumors [2-4] and 82 hematological malignancies have been the subject of a total of 23 studies and case reports, with a clinical response to WT1 in 54% of cases [5]. One of the obstacles for the use of synthetic peptides is that patient accrual is limited to patients with the specific HLA type, for which the peptide is restricted. One strategy that would allow WT1-targeted immunotherapy for patients regardless of their HLA-type, is the transfection of WT1 mRNA into DC to achieve transient expression and subsequent presentation of antigenic epitopes. The *in vitro* work of several laboratories has suggested that mRNA transfection is an effective, if not superior, method of generating immunostimulatory DCs [6-9]. Currently, we evaluate the immunological response following WT1-loaded DC-based immunotherapy in a small cohort of 8 gynecological cancer patients.

MATERIALS AND METHODS

Study design

This was a single-arm, pilot clinical trial (phase I/II) in patients with end-stage uterine cancer, carried out in a single institution (University Hospital Leuven, Belgium; EudraCT 2009-016868-37). Eight gynecological cancer patients, either ovarian cancer of uterine cancer were included. Specific patient

characteristics are summarized in Table 1. All of the included patients were included in the trial suffering from metastasized recurrent disease. All patient received multiple prior treatments. The total number of prior treatment regimens (pooled for both the primary tumor and the recurrence) are shown in Table 1.

DCm-*WT1*-RNA was administered as 4 weekly intradermal injections in imiquimod-treated skin in the groin of the patients. Imiquimod was applied one night before and two nights after vaccination. Patients with disease stabilization or regression received further individually designed DC vaccinations with until disease progression.

The primary end-points were to evaluate the feasibility, toxicity and tumor response rate according to Response Evaluation Criteria in Solid Tumors (RECIST). Secondary end-points were the assessment of the *in vivo* induced immune response and the molecular response of the tumor marker Cancer Antigen 125 (CA125).

Table 1: Patient characteristics.

Patient ID	HLA-A2 status	Tumor type	Histology	prior treatment regimens	Disease status at inclusion	Clinical response after 4 vaccines	Post vaccination therapy	Survival (in days)*
1	positive	EMC	serous	3	PD	PD	chemotherapy	294
2	negative	US	LMS	2	PD	PD	no	111
3	positive	US	LMS	4	PR	SD	boost vaccines	246
4	negative	EMC	serous	6	PD	PD	no	40
5	positive	EMC	serous	4	PD	PD	no	60
6	positive	OS	CS	2	PD	PD	chemotherapy	605
7	positive	US	LMS	2	PD	MR	boost vaccines chemotherapy	218
8	positive	OV	serous	3	PD	PD	hormonal therapy radiotherapy	344**

* survival is expressed in days post WT1 DC vaccination 1

** patient was lost from follow-up. Survival is expressed as days until last follow-up

LMS: leiomyosarcoma, CS: carcinosarcoma, OS: ovarian sarcoma, OV: ovarian carcinoma

Vaccine preparation

For 2/8 patients, the DCm-*WT1*-mRNA vaccine was manufactured in culture flasks. After leukapheresis, monocytes were isolated via plastic adherence and cultured with rhIL-4 (1000 IU/L) and rhGM-CSF (1000 IU/L) in flasks in CellGro cell culture medium + 1 % autologous inactivated plasma. New cytokines and 10 % of medium were added at days 2, 4, and 6. For the remaining 6/8 patients, monocytes were isolated via elutration (Elutra®; Terumo BCT, Lakewood, Colorado, USA) and cultured in closed culture bags (CellGenix, GmbH, Freiburg, Germany) using the same cytokine mixture. After seven days, immature DC (DCi) were harvested for electroporation. DCi were electroporated (300 V, 150 µF exponential decay pulse) to incorporate *WT1*-mRNA

(10 µg per 10 x 10^6 DCi). *WT1*-mRNA used for 7/8 patients was manufactured by CureVac (Tübingen, Germany from a *WT1* plasmid (pGEM4Z-*WT1*-A64)). For patient 8, DC were electroporated with WT1opt-DC.LAMP mRNA (VUB, Brussels, Belgium). Electroporated DC were cultured in the presence of TNF-α (1000 IU/ml) and IL-1β (2000 IU/ml). All cytokines and media were GMP-approved and were purchased from CellGenix (Freiburg, Germany). DCm-*WT1*-mRNA were frozen in liquid nitrogen until vaccination. Prior to vaccination, DC quality was checked by flow cytometry via the expression of maturation markers CD11c and CD83.

Immune monitoring

The immune status and response were assessed at the time of the first vaccine administration (baseline) and at the fourth vaccination (*post* V3). At these time points, PBMC were isolated by Ficoll density gradient centrifugation and cryopreserved in a 20 % HSA solution containing 10% DMSO.

For HLA-A2 positive patients, T cells recognizing the $WT1_{126\text{-}134}$ HLA-A*0201-restricted epitope (RMFPNAPYL) were measured using tetramer staining. PBMC were thawed, resuspended in T cell medium (IMDM + 5% human AB serum + penicillin-streptomycin + L-glutamine, all from Life Technologies, Ghent, Belgium, except for AB serum from Sera Laboratories International Ltd, West Sussex, UK) and left to recover for 1 hour at room temperature in the presence of 10 U/ml DNAse1. Cells were counted with trypan blue and 5 x 10^5 to 1 x 10^6 cells were used for tetramer staining. Cells were resuspended in PBS containing 0.5% BSA and 10% normal goat serum and incubated with PE-labeled WT1 or influenza tetramers (Glycotope Biotechnology, Heidelberg, Germany) for 45 min at 4°C, after which FITC-labeled anti-CD8 (BD Biosciences, San Jose, USA) and PerCp-Cy5.5-labeled anti-CD3 (Biolegend, San Diego, USA) were added.

In parallel, WT1-specific T cells were detected for both HLA-A2 positive and HLA-A2 negative patients using the CD137 assay. In brief, PBMC were thawed as described above and counted with trypan blue. PBMC were resuspended in T cell medium at a concentration of 10^7 cells/ml and incubated with an overlapping peptide mixture covering WT1 (PepTivator, MiltenyiBiotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. Cultures were incubated overnight at 37°C and 5% CO_2, after which cells were harvested and analyzed by flow cytometry using FITC-labeled anti-CD4 (BD Biosciences), PE-labeled anti-CD137 (BD Biosciences), PerCp-Cy5.5-labeled anti-CD3 (Biolegend) and APC-H7-labeled anti-CD8 (BD Biosciences). Results are expressed as fold increase after stimulation with the specified antigen (overlapping peptide pool) compared to mock stimulation. A two-fold increase is considered to be positive. The culture supernatant was stored at -20°C for cytokine quantification. Production of IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10 was quantified using the BD Cytometric Bead Array (CBA, BD Biosciences) according to manufacturer's instructions and analyzed on a FACSCantoII flow cytometer using FCAP Array software (BD Biosciences). In addition, natural killer (NK) cells were measured using PE-conjugated anti-CD161 (Biolegend), PerCp-Cy5.5 conjugated anti-CD16 (BD Biosciences) and PE-Cy7 conjugated anti-CD56 (Biolegend). Abs were added and incubated for another 30 min at 4°C. Subsequently, cells were washed in PBS containing 0.5% BSA and a minimum of 10^5 cells was analyzed by flow cytometry using a FACSort or FACSCanto II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Treestar Inc, Ashland, USA).

RESULTS

Toxicities

No toxicities were reported, evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE), except for local redness of the skin at the injection site. One patient mounted an allergic reaction to the imiquimod cream. For this patient, PGE_2 was added to the DC culture to achieve full DC maturation.

Clinical response

In 2/8 patients, a clinical response could be observed after 4 vaccines. Vaccination resulted in disease stabilization in one patient and a mixed response was seen in one other patient. These 2 patients received additional boost vaccines. No clinical improvement could be seen in any of the 6 other patients (Table 1).

Immune monitoring

In the current cohort, applying the commonly upheld threshold of > 2-fold increase, an immune response was seen in 5/8 patients.
With regard to WT1-specific T cells, tetramer analysis showed a clear WT1-specific T cell response in 1/5 analyzed HLA-A2 positive patients (2.8-fold increase, Table 2). One other patient showed a minor WT1-specific response, yet not reaching the threshold (1.7-fold increase). That same patient also showed an increased $CD8^+CD137^+$ T cell frequency following *in vitro* stimulation with WT1 peptides, further supported by an increased IL-2 secretion. This indicates that patient 7 has an increased frequency of WT1-specific T cells after 4 vaccinations, but these are still in a non-activated state *in vivo.*

Table 2: Immune monitoring.

Patient	HLA-A2 status	$WT1^+$ T cells	CD137 expression		Activated T cells	Activated NK cells	Cytokine levels			
			CD4+	CD8+			IFN-γ	TNF-α	IL-2	IL-10
1	+	**2,88**	-	-	-	-	-	-	-	-
2	-	NA	0,56	0,43	0,75	0,85	ND	ND	ND	ND
3	+	-	-	-	-	-	-	-	-	-
4	-	NA	0,19	0,45	0,92	0,95	ND	ND	ND	ND
5	+	0,45	-	-	-	**2,82**	-	-	-	-
6	+	0,72	0,4	1,27	0,87	0,81	0,13	**2,15**	**6,58**	1,91
7	+	1,73	1,25	**2,8**	0,80	0,49	0,058	0,87	**2,36**	1,04
8	+	0,75	1,80	0,47	0,84	1,19	**20,49**	**25,45**	0,078	**5,64**

NA: not applicable; ND: not detectable; -: not analyzed.
All values are calculated as V4/V1 ratio (fold change of V4 compared to V1)
Values above commonly applied threshold of > 2-fold increase at V4 compared to at V1 are indicated in bold.

In patient 5, a 2.8 fold increase in activated NK cells was seen after 3 vaccinations and patient 6 showed an increased state of *in vitro* immune activation, following stimulation, exemplified by increased secretion of IL-2 and the pleiotropic immune activator TNF-α. The latter was also increased in patient 8, as well as IL-10 and IFN-γ. This patient also showed increased levels of $CD4^+CD137^+$ T cells, indicating a possible presence of an increased frequency of WT1-specific Th1 cells.

DISCUSSION

We present the first series of eight patients with ovarian or uterine cancer treated with autologous DC loaded with WT1 mRNA. This approach was feasible and well tolerated. No side-effects were reported, apart from local redness of the skin at the injection site in all patients and an allergic reaction to the imiquimod cream in one patient. An immunological response was detected in 5/8 patients and 2 patients showed a transient clinical response according to the RECIST criteria. HLA-A2-negative patients did not show an oncological or immunological response. For comparison, Ohno et al. reported two uterine cancer patients receiving WT1 peptide immunotherapy, both showing progressive disease [4]. Miyatake et al. recently reported five patients with uterine sarcoma receiving WT1 peptide immunotherapy, showing disease stabilization in one patient [3]. In these studies, immune monitoring was limited to a test for delayed-type hypersensitivity (DTH) reaction specific to the WT1 peptide used for vaccination. No match between immunological and clinical response was found. Taking together all published series and case reports, in 54.5% of cases, the immunological response matched the clinical response [5].

The limited clinical responses in our study can partly be explained by the condition of the patients included. A large tumor burden, present in four patients, creates an immunosuppressive environment, which hampers the efficacy of immunotherapy. Moreover, the immune system was heavily challenged in nearly all patients because of prior chemotherapy and surgery. Therefore, the immune system may not have fully recovered prior to the immunotherapy regimen.

As in our trial, Miyatake et al. recorded longer survival because of the addition of WT1 immunotherapy [3]. Comparing the survival of the first HLA-A2-positive patient with that of comparable historical controls in University Hospital Leuven shows a striking difference in survival from the moment palliation was declared: 11 months versus 67 days. The same was true for the second HLA-A2-positive patient with end-stage disease. Four comparable historical cases were available in our database. In this small group, two patients died of progression during chemotherapy, one patient before chemotherapy could be initiated, but one patient had a similar cancer history as our patient. She died five months after she was put on palliative care, whereas our patient survived for 10 months. From these observations, it seems that a combination of immunotherapy and chemotherapy might be beneficial, as shown by others [10]. Doxorubicin, for example, results in apoptotic tumor cell death, which increases the antigen uptake by the antigen-presenting cells, leading to in situ immunization against tumor antigens. Chemotherapy might also lead to direct activation of DC, previously demonstrated for doxorubicin and paclitaxel, or their effector mechanisms [11]. Lower doses of chemotherapy, e.g. cyclophosphamide, have been shown to be synergistic with immunotherapy in mice, by depleting regulatory T-cells and retaining memory T-cells [12]. In our study population, there was a clear discrepancy between HLA-A2-positive and -negative patients. HLA type is a known prognostic factor in some types of cancer. However, to our knowledge, nothing has been demonstrated about the HLA type and the response to immunotherapy, nor of a possible dominance of the A2 epitope of WT1. Larger studies are needed to clarify this.

In conclusion, this study presents promising results of the first eight patients with ovarian or uterine cancer to be treated with autologous DC loaded with WT1 mRNA. The technique is feasible and safe. In addition, an immune response following vaccination could be seen in 5/8 patients either *in vivo* or

following *in vitro* activation. In addition, a transient clinical response after 4 vaccinations, was seen in 2/8 patients.

REFERENCES

1. Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, et al. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15(17):5323-37.
2. Coosemans A, Wolfl M, Berneman ZN, Van Tendeloo V, Vergote I, Amant F, et al. Immunological response after therapeutic vaccination with WT1 mRNA-loaded dendritic cells in end-stage endometrial carcinoma. Anticancer research. 2010;30(9):3709-14.
3. Miyatake T, Ueda Y, Morimoto A, Enomoto T, Nishida S, Shirakata T, et al. WT1 peptide immunotherapy for gynecologic malignancies resistant to conventional therapies: a phase II trial. Journal of cancer research and clinical oncology. 2013;139(3):457-63.
4. Ohno S, Kyo S, Myojo S, Dohi S, Ishizaki J, Miyamoto K, et al. Wilms' tumor 1 (WT1) peptide immunotherapy for gynecological malignancy. Anticancer research. 2009;29(11):4779-84.
5. Van Driessche A, Berneman ZN, Van Tendeloo VF. Active specific immunotherapy targeting the Wilms' tumor protein 1 (WT1) for patients with hematological malignancies and solid tumors: lessons from early clinical trials. The oncologist. 2012;17(2):250-9.
6. Erdmann M, Dorrie J, Schaft N, Strasser E, Hendelmeier M, Kampgen E, et al. Effective clinical-scale production of dendritic cell vaccines by monocyte elutriation directly in medium, subsequent culture in bags and final antigen loading using peptides or RNA transfection. Journal of immunotherapy. 2007;30(6):663-74.
7. Gilboa E, Vieweg J. Cancer immunotherapy with mRNA-transfected dendritic cells. Immunological reviews. 2004;199:251-63.
8. Michiels A, Tuyaerts S, Bonehill A, Corthals J, Breckpot K, Heirman C, et al. Electroporation of immature and mature dendritic cells: implications for dendritic cell-based vaccines. Gene therapy. 2005;12(9):772-82.
9. Van Tendeloo VF, Ponsaerts P, Lardon F, Nijs G, Lenjou M, Van Broeckhoven C, et al. Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. Blood. 2001;98(1):49-56.
10. Antonia SJ, Mirza N, Fricke I, Chiappori A, Thompson P, Williams N, et al. Combination of p53 cancer vaccine with chemotherapy in patients with extensive stage small cell lung cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2006;12(3 Pt 1):878-87.
11. Shurin GV, Tourkova IL, Kaneno R, Shurin MR. Chemotherapeutic agents in noncytotoxic concentrations increase antigen presentation by dendritic cells via an IL-12-dependent mechanism. Journal of immunology. 2009;183(1):137-44.
12. Kandalaft LE, Singh N, Liao JB, Facciabene A, Berek JS, Powell DJ, Jr., et al. The emergence of immunomodulation: combinatorial immunochemotherapy opportunities for the next decade. Gynecologic oncology. 2010;116(2):222-33.

Part V

Chapter 7 Variability in CRP, regulatory T cells and effector T cells over time in gynecological cancer patients: a study of potential oscillatory behavior and correlations

---

Madondo M, Tuyaerts S, Turnbull B, Scalzo-Inguanti K, **Vanderstraeten A**, Kohrt H, Narasimhan B, Amant F, Quinn M, Plebanski M.

Selected, adapted and extended from Madondo M et al. Variability in CRP, regulatory T cells and effector T cells over time in gynaecological cancer patients: a study of potential oscillatory behaviour and correlations. J Transl Med. 2014; 23;12(1):179.

Variability in CRP, regulatory T cells and effector T cells over time in gynecological cancer patients: a study of potential oscillatory behavior and correlations

ABSTRACT

The inflammatory marker, C-reactive protein has been proposed to also be a biomarker for adaptive immune responses in cancer patients with a possible application in time based chemotherapy. Fluxes in serum C-reactive protein levels were suggested to be indicative of a cyclical process in which, immune activation is followed by auto-regulating immune suppression. The applicability of C-reactive protein as a biomarker for regulatory or effector T cells was therefore investigated in a cohort of patients with gynecological malignancies, as well as the ability of low dose cyclophosphamide to influence the frequencies of both these cell types.

Peripheral blood samples were obtained from a cohort of patients at 7 time points over a period of 12 days before treatment and at 3 time points after each cycle of cyclophosphamide. Serum and mononuclear cells were isolated and C-reactive protein levels in serum were detected using ELISA while regulatory and effector T cell frequencies were assessed using flow cytometry. To test periodicity, periodogram analysis of data was employed while Pearson correlation and the Wilcoxon signed rank test were used to determine correlations.

The statistical analysis used showed no evidence of periodic oscillation in either serum C-reactive protein concentrations or frequencies. Furthermore, there was no apparent correlation between serum CRP concentrations and the corresponding frequencies of regulatory T cells or effector T cells. Relative to healthy individuals, the disease state in the patients neither significantly affected the mean frequency of regulatory T cells nor the mean coefficient of variation within the regulatory T cell population over time. However, both effector T cell mean frequency and mean coefficient of variation were significantly reduced in patients. No effects of cyclophosphamide treatment on Treg frequencies could be observed.

Using our methods we were unable to detect C-reactive protein oscillations that could be used as a consistent serial biomarker for time based chemotherapy.

INTRODUCTION

The link between inflammation and tumor development has been widely reported and recently, in an update to their seminal paper on the hallmarks of cancer, Hanahan and Weinberg refer to inflammation as an “enabling characteristic” for tumor development [1]. This link is well illustrated in gynecological malignancies. The processes of ovulation and menstruation are associated with inflammation of the ovarian surface and endometrial epithelia [2, 3]. During ovulation there is an increase in the macrophage population in the perifollicular stroma of pre-ovulatory follicles [4, 5]. These macrophages produce pro-inflammatory cytokines interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF), [2]. Estrogen in the endometrium also induces the up-regulation of these pro-inflammatory cytokines [6]. Over time, the cyclical process of epithelial damage and repair, involving inflammation, predisposes to neoplastic growth. Not only does inflammation play a role in tumorigenesis, it also facilitates disease progression. The tumor microenvironment is rich in inflammatory mediators produced by tumor cells and infiltrating leukocytes [7-9]. Specifically, IL-6

triggers tumor cells to produce matrix metalloproteinase (MMP)-9 that promotes tumor growth by initiating angiogenesis [10].

C-reactive protein (CRP), an acute phase protein produced by hepatocytes is the most widely used indicator of inflammation. The transcription of CRP is induced by IL-6 alone while the presence of either IL-1β or TNF augments its production [11, 12]. Gynecological cancer patients exhibit elevated levels of CRP, however these levels are not static. In a preliminary study of melanoma and ovarian cancer patients, serum CRP levels oscillated around a certain mean value with a periodicity (λ) of 7 days [13]. The dynamic state of inflammation could indicate an underlying persistent but regulated antitumor immune response in the cancer patients, which is characterised by a cyclical repeating process of endogenous auto-vaccination, preceding suppression by regulatory cells.
In the past few decades the role of the adaptive immune system in driving antitumor immunity has been highlighted. Studies have shown a positive correlation between the number of tumor-infiltrating effector T cells and patient survival, as well as a negative correlation with $Foxp3^{+}CD25^{Hi}CD4^{+}$ regulatory T cells [14, 15]. Inflammatory cytokines and chemokines such as TNF and CCL22 facilitate the infiltration of T cells into the tumor microenvironment. When activated, effector T cells up-regulate CD25 and transiently express FoxP3 [16]. This enables them to expand and secrete pro-inflammatory cytokines. $FoxP3^{+}CD25^{Hi}CD4^{+}$ regulatory T cells, which express high levels of the CCL22 receptor CCR4, also infiltrate tumor sites where they expand in response to TNF and IL-2 produced by effector T cells, and subsequently inhibit the immune response [17-19].
Commonly used chemotherapy has been shown to have immunomodulating effects. Thus, the use of these compounds may have a beneficial effect on tumor immune escape. Cyclophosphamide, for example, has been shown to influence the frequency and functional properties of regulatory T cells. Both the presence of immune-oscillation patterns of CRP, regulatory and effector T cells was evaluated in this study, as well as the influence of oral low-dose CY intake on the frequency and functional capacities of these cell types.

METHODS

Trial design and patient details

In total, 19 patients with gynecological tumors were included in this bicentric phase II trial. The patients had end stage treatment-refractory gynecological tumors, i.e. ovarian cancer (n=5), papillary serous cystadenocarcinoma (n=9), adenocarcinoma (n=1), serous cystadenocarcinoma (n=1), endometrial carcinoma (n=1), peritoneal tumor, (n=1) or malignant mixed müllerian tumors (n=1) and were recruited from the Royal Women's Hospital, Melbourne, Australia (n=12) and the University Hospital Leuven, Belgium (n=7). Patient age ranged from 43-82 years. The relative institution's research and ethics committees approved the study. From the time of recruitment, written consent was obtained from patients and 7 blood collections were performed over 12 days with daily and second daily bleeds, to assess the presence of a CRP cycle. Blood was collected in the morning on days 1, 3, 5, 6, 8, 10 and 12 with one exception who had blood collected on days 1, 2, 4, 5, 6, 8, 12. Following collection, the blood samples were immediately transported to the laboratory for processing with a maximum of 12 hours between blood collection and freezing of plasma. Based on predicted CRP cycling patterns [13], patients went on to receive treatment with low-dose cyclophosphamide, 50 mg administered p.o. twice daily for 3 consecutive days. Blood was also collected from 7 healthy volunteers at the Alfred Hospital, Melbourne, Australia. Ethical approval was

obtained from the institution's research board and written consent was also obtained from the volunteers. The blood collection protocol for the healthy volunteers was similar to that of the cancer patients. Specific characteristics of the all patients are summarized in Table 1.

Table 1: Patient characteristics.

Patient ID	Age	Tumor type	Prior Treatment	Disease status at inclusion
BLV 01	74	Ovarian carcinoma	Surgery, 3 lines chemo	PD with newly formed hepatic lesion, hepatogastric, lymph node, peritoneal and mesenteric lesions present
BLV 02	80	Ovarian carcinoma	Surgery, 3 lines chemo, provera	PD with increase of peritoneal metastases with ascites formation
BLV 03	73	Ovarian carcinoma	3x surgery, 4 lines chemo	PD with increase and new formation of abdominal metastases and possible lymph node metastases
BLV 04	64	Peritoneal tumor	Surgery, 6 lines chemo, avastin	PD with peritoneal, omental, vertebral, hepatic and possible gastric metastases
BLV 05	62	Endometrial carcinoma	2x surgery, 1 line chemo, radiotherapy	PD with increase of hepatic metastases and possible renal metastases, as well as brain metastases
BLV 06	74	Malignant mixed Müllerian tumor (MMMT)	Surgery, 1 line chemo	PD with increase of known retroperitoneal lesions and occurence of a new lesion
BLV 07	82	Ovarian carcinoma	2x surgery, 5 lines chemo, provera	PD with increase of LN metastases in upper abdomen, metastases in mediastinum and formation of lung metastases
IRS 01	43	Adenocarcinoma	Surgery, 1 line chemo, radiotherapy and EGFR inhibitor	PD with recurrence in supraclavicular fossa lymph node
IRS 02	45	Ovarian carcinoma	Surgery, 3 lines chemo, radiotherapy	PD with hepatic involvement
IRS 03	54	Papillary serous cystadenocarcinoma	Surgery, 2 lines chemo, radiotherapy	PD with rising CA-125 marker
IRS 04	63	Papillary serous cystadenocarcinoma	Surgery, 5 lines chemo, hormone therapy	PD with rising CA-125 marker
IRS 05	68	Serous cystadenocarcinoma	2 x Surgery, 1 line chemo, hormone therapy	PD with rising CA-125 marker
IRS 06	74	Papillary serous cystadenocarcinoma	Surgery, 2 lines chemo	PD rising CA-125 marker , PAN and adrenal gland involvement
IRS 07	66	Papillary serous cystadenocarcinoma	Surgery, 3 line chemo, radiotherapy, 1 x EGFR inhibitor	PD with rising CA125
IRS 08	63	Papillary serous cystadenocarcinoma	Surgery, 2 lines chemo	PD with rising CA125 level
IRS 09	63	Papillary serous cystadenocarcinoma	Surgery, 5 lines chemo	PD with omental mass increased in size
IRS 11	70	Papillary serous cystadenocarcinoma	Surgery, 2 lines chemo	Progessive disease (PD) with rising CA125
IRS 12	79	Papillary serous cystadenocarcinoma	Surgery, 2 lines chemo	PD with progressive nodal abnormalities above and below diaphragm and new peritoneal abdo/pelvic disease
IRS 13	69	Papillary serous cystadenocarcinoma	Surgery, 2 lines chemo	PD with short bowel syndrome

hsCRP plasma level measurements

Plasma was isolated from whole blood collected either in EDTA-coated tubes (Belgian patients) or in serum separation tubes (Australian patients) by centrifugation. After removing cellular and protein debris, plasma was aliquoted and stored at -80°C for later use. HsCRP levels were determined by ELISA (Human High Sensitivity C-Reactive Protein ELISA kit; Cusabio Biotech Co., LTD, China) according to manufacturer's instructions. For each treatment cycle, batch analysis was performed on all the necessary samples. To increase experimental precision, all samples were analyzed in duplicate.

Peripheral blood mononuclear cell isolation

Mononuclear cells were obtained from peripheral whole blood collected in EDTA-coated tubes via Ficoll (Amersham Pharmacia Biotech, Sweden) density gradient centrifugation. The isolated PBMCs were cryopreserved using a freeze mixture containing 10% DMSO (Sigma-Aldrich, Australia) and either 90% Fetal Calf Serum (Australian samples, JRH Bioscience) or human AB serum (Belgian samples, Sera Laboratories International) and stored in freezing containers (Nalgene) and finally in liquid nitrogen until use. For use, cells were thawed in a 37°C water-bath and quickly re-suspended using AIM-V medium (Invitrogen) with 5% human serum (Sigma). PBMC samples were available at all time points from 10 patients in total.

Flow cytometric analysis
(7/10 analyzed patients are included at University Hospital Leuven)

To determine the frequency and phenotype of T cell populations in PBMC of patients with gynecological malignancies and age-matched female, healthy volunteers, multicolor flow cytometric analysis was performed using the following surface antibodies: anti-CD3 Q655 (Invitrogen), anti-CD4 AF700 (BD Pharmingen), anti-CD25 PE (BD Pharmingen), and anti-CD127 Biotin (BD Pharmingen). Following primary staining, a fixable dead cell dye (Invitrogen) was also used to distinguish between dead and live cells. Intracellular levels of FoxP3 were determined following fixation and permeabilization using a fixation/permeabilization buffer kit (eBioscience) then staining with anti-FoxP3 PercpCy5.5 (eBioscience). Flow cytometry data were acquired on a Becton Dickinson LSR II using FACSDiva software, collecting a minimum of 100 000 events per sample. Fluorescence minus one (FMO) and isotype-matched antibodies were used as controls with all samples. Data were analyzed using Flowjo software (TreeStar). Samples were analyzed applying a gating strategy as follows: first, live PBMC were selected. From live PBMC, $CD3^+$ T cells were gated, after which $CD4^+$ and $CD8^+$ cells were separately selected. Within the $CD4^+$ cell population, $CD25^{HI}$ and $CD25^{int}$ cells were identified. FoxP3 expression was analyzed on $CD4^+CD25^{HI}$ cells

FOXP3 PCR
(all patients are included at University Hospital Leuven)

Genomic DNA (gDNA) was isolated from PBMC pellets using the Purelink™ Genomic DNA Mini kit (Invitrogen). In order to discriminate the methylated and non-methylated form of FOXP3 a bisulfite reconversion was subsequently performed using the Epitect® Plus DNA Bisulfite kit (Qiagen). Analysis was performed using primers and probes specific for (un)methylated FOXP3 (Eurogentec) according to the following program: 10' at 95°C followed by 45 cycles of 15'' at 95°C and 1' at 64°C. The exact primer and probe sequences for both forms of FOXP3 are given in Table 2. Results are analyzed as

follows: total copy number of both methylated and demethylated sequences were calculated based on a plasmid standard curve multiplied with a correction factor of 1 for methylated and 0.3 for demethylated FOXP3. Final results are expressed as % demethylated sequences out of the total number of sequences, corrected for the number of chromosomes. In this case a correction factor of 2 was used since the gene encoding FOXP3 is located on the X chromosomes and the entire patient population is female.

Table 2: Primer/probe sequences for the detection of methylated and demethylated FOXP3 sequences.

	Methylation pattern	**sequence**	**LNA modified bases**
Probe	methylated	AAACCC**G**A**CG**CATCCGAC	3
FW primer	methylated	CTCTTCTCTTCCTCCGTAATATCG	
RV primer	methylated	GTTATTGACGTTATGGCGGTC	
Probe	demethylated	AAACC**CA**A**CAC**ATCCAACCA	5
FW primer	demethylated	TCTACCCTCTTCTCTTCCTCCA	
RV primer	demethylated	GATTTTTTTGTTATTGATGTTATGGT	

All LNA modified bases are underlined and indicated in bold.

Statistics

To determine the respective periodicity of serum CRP concentration, and Teff and Treg frequencies periodogram analysis was used. The null hypothesis was that there was no consistent period to the measurements, which implies no peaks in the mean periodogram beyond noise. Individual subjects' periodograms were calculated and standardized to have sum of squares equal to 1, then averaged pointwise. Under the null hypothesis, the population pointwise mean periodogram is a horizontal line at 1. The pointwise, lower one-sided 95% confidence bound for the mean periodogram was calculated by the bias-corrected bootstrap method. This lower confidence bound was then compared to the null mark at 1. Exceeding 1 at a peak would be necessary to suggest a significant peak.

The Pearson correlation coefficient between Teff first differences and CRP first differences was estimated for each subject, and the Wilcoxon signed rank test was used to test whether the mean correlation coefficient was non-zero. The same procedure was performed to test for a relationship between Treg and CRP, Tregs and Teffs, and CRP and IL-6. The first difference is defined as the change in value from one time point to the next.

Coefficients of variation in the frequencies of Tregs and Teffs among 7 time points over a period of 12 days were determined by initially calculating the standard deviation, which was then expressed as percentages of the mean frequency of the respective population over the 7 time points. Statistical significances between mean values of Treg and Teff frequencies as well as coefficients of variation for both populations were calculated using non-parametric (Wilcoxon Mann Whitney) tests. P values < 0.05 were considered significant. All mean values were presented ± the standard error of the mean (SEM).

Statistical significance was determined by performing 2 tailed, unpaired students' t-Tests for comparison of healthy and cancer patient samples. Two tailed, paired Students t-Tests were performed for comparison of patients' pre-treatment and post-treatment samples. P<0.05 was

considered to be significant. The software used for statistical analysis was GraphPad Prism. Data was always shown as mean ± Standard Error from the Mean (SEM).

RESULTS

Oscillation patterns of CRP

In the individual patients serum hsCRP concentrations appeared to fluctuate over time (Fig 1A). However, when a mean standard periodogram analysis was carried out for the pooled patient cohort at every time point, while small peaks were apparent in the periodograms at 3.6 days and 4.6 days, neither was significant (Fig 1B). In a smaller cohort of 10 patients from whom PBMCs were collected, the periodicity of peripheral blood Treg and Teff frequencies was also investigated. Tregs were defined as expressing high levels of CD25 and FoxP3, which we confirmed expressed low levels of CD127, while Teffs were defined as expressing intermediate levels of CD25 as per previous studies [20, 21]. Similar to serum CRP concentration, both frequencies appeared to fluctuate over time, in some individual patients (data not shown). In the pooled cohort, mean standard periodogram analysis revealed small peaks for Treg frequencies at 4.8 days and for Teff frequencies at 3.6 and 4.6 days, but none was significant (data not shown).

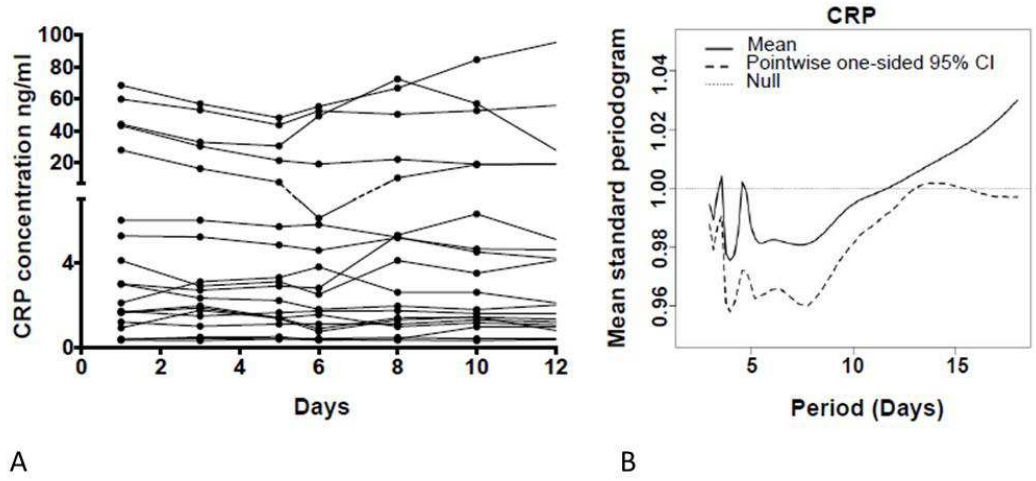


Figure 1: CRP cycle analysis. A. hsCRP analysis. B. Periodogram analysis.

Effect of cyclophosphamide treatment on T cell distribution

We investigated the effect of treatment with repeated cycles of low dose cyclophosphamide (CY) on the distribution of T cells in peripheral blood of 10 end-stage gynecological cancer patients and further used 11 healthy, age-matched, female donors as controls. In 8/10 patients, samples were available following the second line of CY treatment. There was no significant effect of treatment on the overall frequencies of $CD3^+$ T cells within peripheral blood of patients, nor in the frequencies of $CD4^+$ and $CD8^+$ T cells (Fig. 2A, B). Compared to pre-treatment levels in cancer patients, there was a significant increase in the mean frequency of $CD4^+CD25^+$ cells (both $CD25^{Hi}$ and $CD25^{Int}$) following the first cycle of treatment ($p < 0.05$; Fig. 2C). Furthermore, the mean frequency of $CD25^+FoxP3^+$ cells as well as FoxP3 expression within the $CD25^{Hi}CD4^+$ T cell population was significantly higher in patients than in healthy donors prior to treatment as well as following CY treatment. However, the

frequencies of FoxP3^{+}CD25^{Hi} regulatory T cells (Tregs) within CD4^{+} T cells were not significantly different between healthy donors and cancer patients following either treatment cycle (Fig 2D).

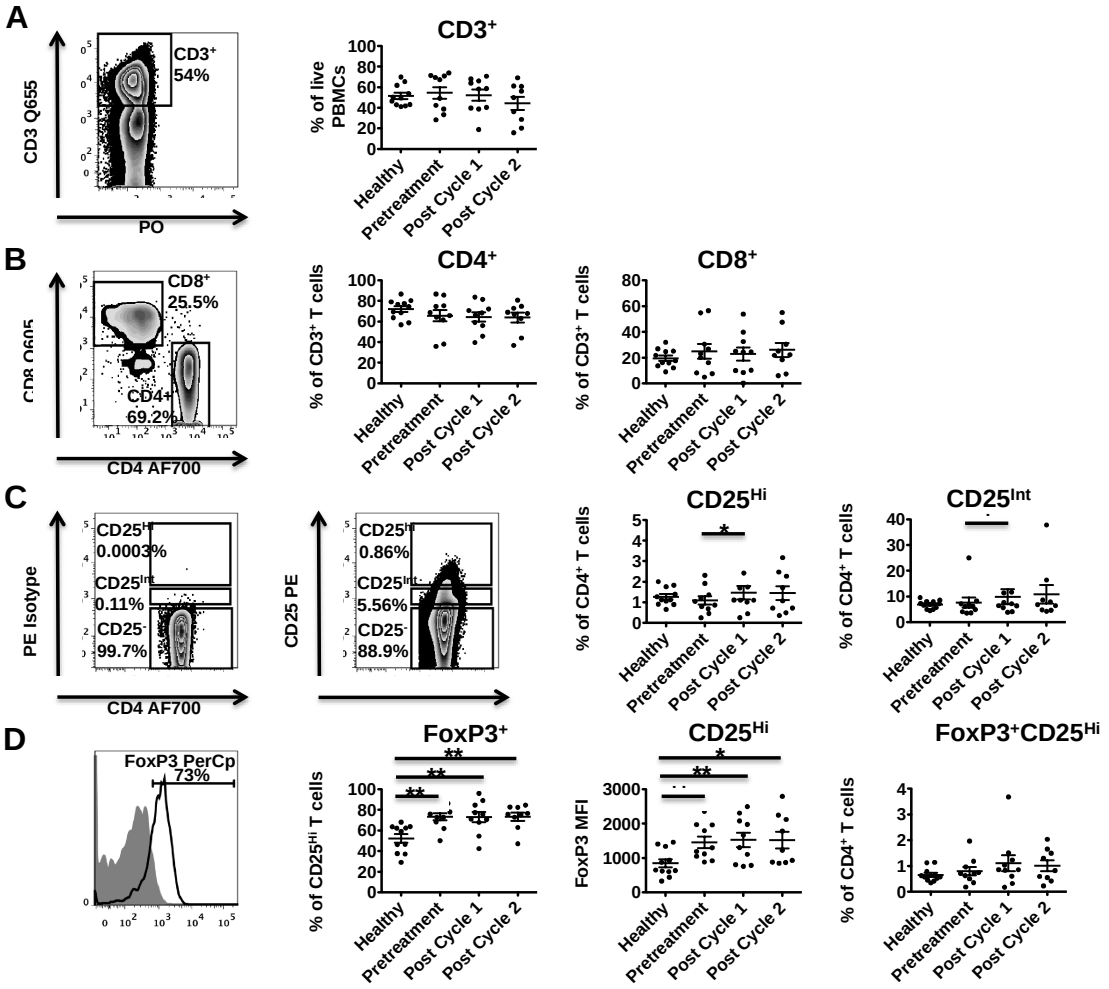


Figure 2: A. CD3^{+} T cell frequencies in peripheral blood. B. CD4^{+} and CD8^{+} T cell frequencies. C. CD25 expression within the CD4^{+} T cell population. D. FoxP3 analysis. All cycles were compared with healthy controls using the student's t test.

Correlation between FoxP3^{+} T cell frequencies and FOXP3 mRNA transcripts

In conjunction with the frequency of Treg in peripheral blood, the level of demethylated FOXP3 in PBMC were analyzed. As shown in Fig. 3, although the Treg frequencies were different with both methods, the fluctuation pattern was similar using both assays. However, we did notice that the fluctuation pattern of both parameters did not occur simultaneously across the entire period of analysis (Fig. 3).

Effect of cyclophosphamide treatment on T cell effector functions

To additionally investigate whether treatment may enhance antitumor immunity by increasing CD8^{+} T cell cytotoxic function, PBMC collected from 7 healthy donors and 6 cancer patients, prior to treatment, and after cycles 1 and 2 of treatment were stimulated with PMA /Ionomycin. Contrary to expectation, compared to healthy donors, cancer patients had a slightly elevated mean frequency of CD8^{+}IFN-γ^{+}T cells and this was significantly higher following the second cycle of treatment (20.3%±4.06% compared to, 39.7%±7.81% p = 0.04; Fig. 4A). Considering the cancer patients

pre-treatment, there were no significant differences in the frequencies of $CD8^{+}IFN\text{-}\gamma^{+}$ T cells following either cycle of treatment (Fig 4B). Conversely, the frequency of $CD107a^{+}$ cells remained constant between healthy donors and cancer patients, and was not affected by treatment (Fig 4B).

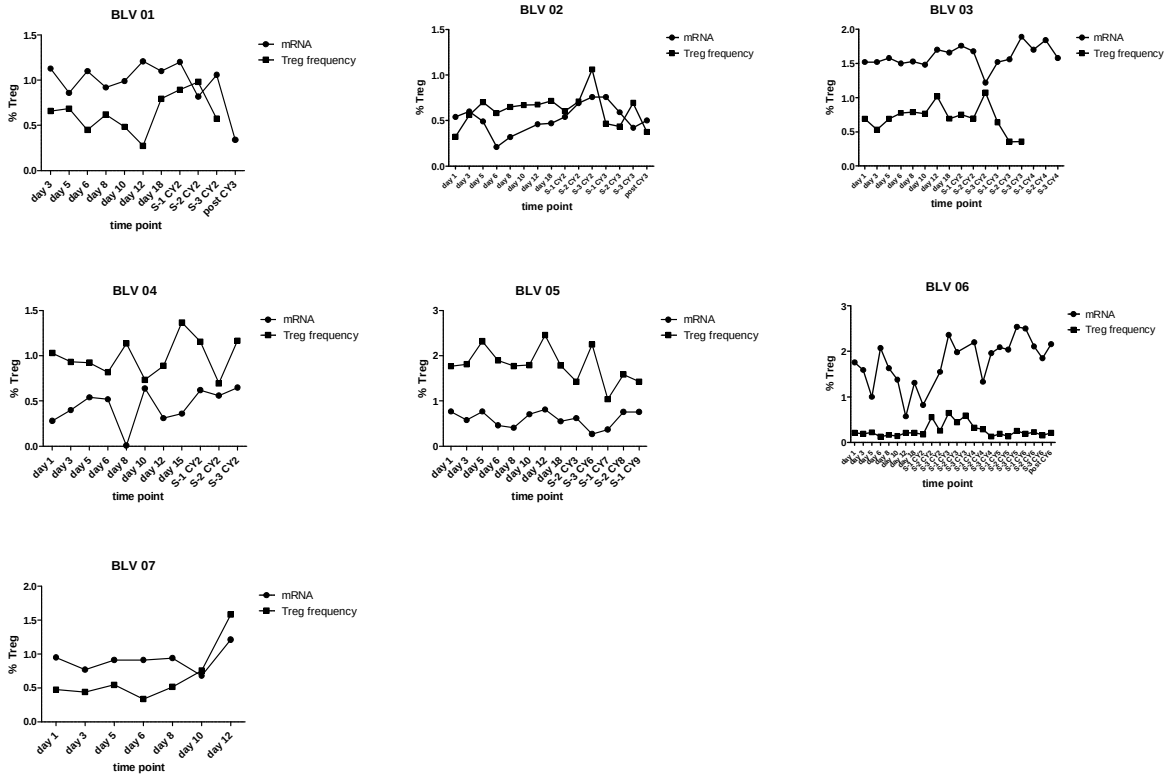


Figure 3: Treg frequencies by flow cytometry and FoxP3 mRNA levels in PBMC.

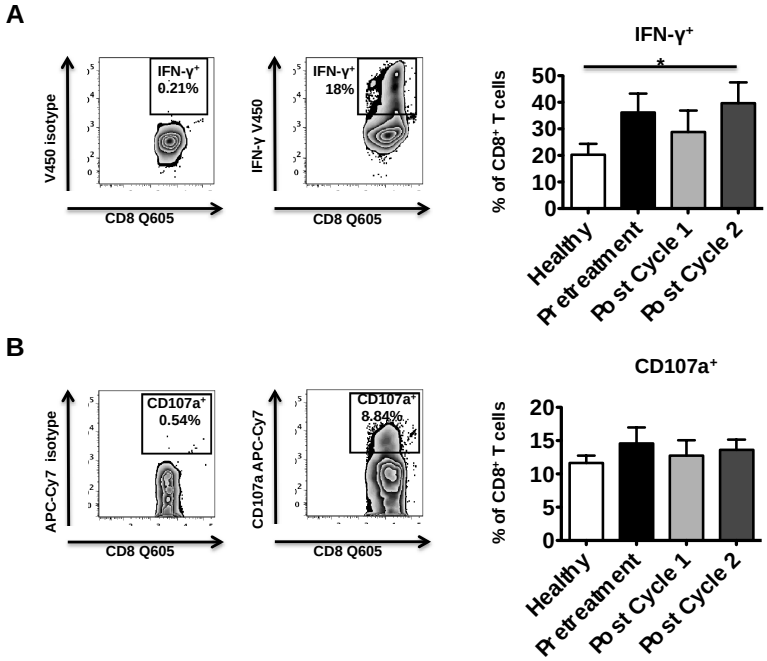


Figure 4: Effect of cyclophosphamide treatment on T cell effector functions. A. IFN-γ secretion. B. CD107a expression. All cycles were compared with healthy controls using the student's t test.

Low-dose cyclophosphamide and treatment response

Prior to treatment and after each treatment cycle, CA125 measurements were done and a CT scan was performed for clinical evaluation of the low-dose cyclophosphamide treatment every 3 cycles. Although all patients eventually progressed (Table 3), 7/19 (36.8 %) patients received multiple cycles of cyclophosphamide, indicating transient clinical responses.

Table 3: Clinical response data.

Patient ID	**Disease state at inclusion**	**Total number of cycles received**	**CA125 evolution**	**Target-related adverse events**
IRS001	PD	15	Transient decrease	-
IRS002	PD	3	Increase	-
IRS003	PD	2	Increase	-
IRS004	PD	9	Stable after 3 cycles	-
IRS005	PD	1	-	-
IRS006	PD	6	Fluctuating	-
IRS007	PD	3	Increase	-
IRS008	PD	5	Transient stabilization	-
IRS009	PD	2	Increase	-
IRS011	PD	2	Increase	-
IRS012	PD	2	Increase	-
IRS013*	PD	6	Fluctuating	-
BLV01	PD	3	Increase	-
BLV02	PD	3	Increase	-
BLV03*	PD	3	Increase	-
BLV04	PD	2	Increase	-
BLV05	PD	9	Transient decrease	-
BLV06	PD	6	Stable until CY6	-
BLV07*	PD	2	Increase	-

** dropped out of the study at patients' own request*

DISCUSSION

The dynamic nature of the adaptive immune response has been described in two ways. Firstly, there are diurnal fluctuations, regulated by the circadian clock exemplified in $CD4^+$ T cells by the rhythmic expression of genes that control cytokine secretion and cell function [22]. Secondly, antigen dependent fluctuations occur during acute infections and the kinetics of the immune response are controlled by a system of positive and negative feedback mechanisms designed to limit the immune response when pathogenic insults are resolved. In chronic diseases such as cancer, antigenic clearance does not occur and the persistent antigen exposure results in a constant state of immune activation. The tumor, however, limits immune activation by secreting immune inhibitory cytokines such as IL-10, and tumor growth factor (TGF)-β as well as inducing regulatory cell populations including myeloid derived suppressor cells (MDSCs) and regulatory T cells [18, 23-25]. Although intratumoral immune cells are skewed towards immune inhibition, there may still exist a homeostatic balance that needs to be maintained in the periphery of cancer patients in which an

oscillating sequence of immune activation is followed by negative feedback immune suppression [13]. The current study however, showed that CRP concentrations in all the patients did not appear to oscillate periodically nor did the concentration of CRP correlate significantly with Treg or Teff frequencies. Therefore, CRP may not be a practical useful surrogate marker for either cell population. In a study of 12 patients with melanoma, less than 50% showed possible time dependent CRP concentration profiles [26]. The two data sets available therefore suggest that inflammation in cancer patients may not consistently follow an oscillatory, sinusoidal (or other mathematical) pattern with a constant period or amplitude. This may be because any such model oversimplifies the inflammatory process, and correlations are easily disrupted by a number of potential additional *in vivo* co-parameters or process variations. Multiple factors influence the levels of cytokines that regulate inflammation. One such factor is the tumor growth. In patients with papillary thyroid cancer $CD4^+$ T cell frequencies correlate with tumor size while Treg frequencies correlate with lymph node metastasis [27]. Such variables may hence significantly influence the magnitude of T cell effector and suppressor frequency and function.
Although changes in the frequencies of conventional Treg and Teff did not correlate with inflammation (data not shown), it is still possible that minor subsets within each phenotype (Treg or Teff) or their specific function over time, may correlate. Indeed, effector and regulatory T cells are heterogeneous populations of cells. Further breaking down these populations based on phenotype and function may also show greater variation between patients and healthy donors. For example, Treg populations with enhanced suppressive function due to elevated expression of inhibitory receptors such as glucocorticoid induced tumor necrosis factor receptor (GITR) and cytotoxic T-lymphocyte antigen (CTLA)-4 as well as increased production of suppressive factors such as adenosine and cytokines TGF-β and IL-10, have been reported to be elevated in cancer patients [28-30]. Similarly, effector T cells can be broken down into different functional phenotypes such as IL-17 secreting and type-1 interferon secreting Teffs. Type-1 interferon secreting Teffs promote proliferation of cytotoxic $CD8^+$ T cells, which contribute toward an antitumor effect [31-34]. It cannot therefore be excluded that the frequencies of some of these functional subsets, as well as $CD8^+$ T cells, may be subject to regulation by inflammatory factors, even when the results presented show that total Treg and Teff populations are not correlated to inflammatory status, as reflected by CRP levels in blood. Mathematical models that aim to predict the balance that exists between immune activation and regulation within accessible blood samples, will therefore benefit from additionally taking into account the following variables: 1) the effect of diurnal variation 2) the tumor growth rate 3) the heterogeneity present within both regulatory and effector T cell populations.

Cyclophosphamide (CY) administration has been shown to result in immunomodulatory effects including selective depletion of regulatory T cells as well as increased effector T cell function [35-38]. However, these effects are not seen in all studies. de Vries et al. evaluated the effect of several possible Treg-depleting agents, among which cyclophosphamide. In this study, none of the agents could induce a 50 % reduction in peripheral blood frequencies in the majority of the patients tested [39]. In our analysis, partly corroborating these results, no effect of cyclophosphamide on Treg depletion could be seen. However, in the current analysis, again, no discrimination was made for the different types of Treg, e.g. inducible and natural Treg. In addition, we were not able to show an increased effector cell function after cyclophosphamide treatment.

In conclusion, in our cohort of patients with gynecological malignancies, CRP concentrations do not oscillate in a consistent predictable manner, and do not correlate either positively or negatively with

conventional Treg or Teff subsets. Therefore, there is no evidence to suggest that CRP can reliably be used across cancer patients as a surrogate, time-sensitive and most importantly, predictive marker, to reflect circulating effector or regulatory T cell frequencies, as previously suggested [13]. Time-based therapy founded on modelling a consistent cyclical pattern of inflammation using serum CRP concentration as a predictive marker of regulatory T cell expansion may not be possible. However, we cannot exclude that further investigating the kinetics of inflammation in cancer patients, perhaps by taking more frequent blood samples or else by taking into consideration multiple inflammatory and immune-regulatory parameters, the progressive nature of immune suppression, as well as the heterogeneity of effector and regulatory T cell populations could all help in modelling more complex, and potentially predictive, equations. Metronomic treatment with low-dose cyclophosphamide did not result in selective Treg depletion nor were any durable clinical responses noticed.

REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
2. Maccio A, Madeddu C. Inflammation and ovarian cancer. Cytokine. 2012;58(2):133-47.
3. Wallace AE, Gibson DA, Saunders PT, Jabbour HN. Inflammatory events in endometrial adenocarcinoma. The Journal of endocrinology. 2010;206(2):141-57.
4. Brannstrom M, Pascoe V, Norman RJ, McClure N. Localization of leukocyte subsets in the follicle wall and in the corpus luteum throughout the human menstrual cycle. Fertility and sterility. 1994;61(3):488-95.
5. Dahm-Kahler P, Ghahremani M, Lind AK, Sundfeldt K, Brannstrom M. Monocyte chemotactic protein-1 (MCP-1), its receptor, and macrophages in the perifollicular stroma during the human ovulatory process. Fertility and sterility. 2009;91(1):231-9.
6. He YY, Cai B, Yang YX, Liu XL, Wan XP. Estrogenic G protein-coupled receptor 30 signaling is involved in regulation of endometrial carcinoma by promoting proliferation, invasion potential, and interleukin-6 secretion via the MEK/ERK mitogen-activated protein kinase pathway. Cancer science. 2009;100(6):1051-61.
7. Charles KA, Kulbe H, Soper R, Escorcio-Correia M, Lawrence T, Schultheis A, et al. The tumor-promoting actions of TNF-alpha involve TNFR1 and IL-17 in ovarian cancer in mice and humans. J Clin Invest. 2009;119(10):3011-23.
8. Kulbe H, Chakravarty P, Leinster DA, Charles KA, Kwong J, Thompson RG, et al. A dynamic inflammatory cytokine network in the human ovarian cancer microenvironment. Cancer Res. 2012;72(1):66-75.
9. Burke F, Relf M, Negus R, Balkwill F. A cytokine profile of normal and malignant ovary. Cytokine. 1996;8(7):578-85.
10. Rabinovich A, Medina L, Piura B, Segal S, Huleihel M. Regulation of ovarian carcinoma SKOV-3 cell proliferation and secretion of MMPs by autocrine IL-6. Anticancer research. 2007;27(1A):267-72.
11. Young DP, Kushner I, Samols D. Binding of C/EBPbeta to the C-reactive protein (CRP) promoter in Hep3B cells is associated with transcription of CRP mRNA. J Immunol. 2008;181(4):2420-7.
12. Yoshizaki K. Pathogenic role of IL-6 combined with TNF-alpha or IL-1 in the induction of acute phase proteins SAA and CRP in chronic inflammatory diseases. Advances in experimental medicine and biology. 2011;691:141-50.
13. Coventry BJ, Ashdown ML, Quinn MA, Markovic SN, Yatomi-Clarke SL, Robinson AP. CRP identifies homeostatic immune oscillations in cancer patients: a potential treatment targeting tool? Journal of translational medicine. 2009;7:102.

14. Li J, Mo HY, Xiong G, Zhang L, He J, Huang ZF, et al. Tumor microenvironment MIF directs the accumulation of IL-17-producing tumor-infiltrating lymphocytes and predicts favorable survival in nasopharyngeal carcinoma patients. J Biol Chem. 2012.
15. Katz SC, Bamboat ZM, Maker AV, Shia J, Pillarisetty VG, Yopp AC, et al. Regulatory T Cell Infiltration Predicts Outcome Following Resection of Colorectal Cancer Liver Metastases. Annals of surgical oncology. 2012.
16. Allan SE, Crome SQ, Crellin NK, Passerini L, Steiner TS, Bacchetta R, et al. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. International immunology. 2007;19(4):345-54.
17. Miller AM, Lundberg K, Ozenci V, Banham AH, Hellstrom M, Egevad L, et al. CD4+CD25high T cells are enriched in the tumor and peripheral blood of prostate cancer patients. J Immunol. 2006;177(10):7398-405.
18. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nature medicine. 2004;10(9):942-9.
19. Hamano R, Huang J, Yoshimura T, Oppenheim JJ, Chen X. TNF optimally activatives regulatory T cells by inducing TNF receptor superfamily members TNFR2, 4-1BB and OX40. Eur J Immunol. 2011;41(7):2010-20.
20. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med. 2006;203(7):1693-700.
21. Triplett TA, Curti BD, Bonafede PR, Miller WL, Walker EB, Weinberg AD. Defining a functionally distinct subset of human memory CD4+ T cells that are CD25POS and FOXP3NEG. Eur J Immunol. 2012;42(7):1893-905.
22. Bollinger T, Leutz A, Leliavski A, Skrum L, Kovac J, Bonacina L, et al. Circadian clocks in mouse and human CD4+ T cells. PloS one. 2011;6(12):e29801.
23. Mustea A, Konsgen D, Braicu EI, Pirvulescu C, Sun P, Sofroni D, et al. Expression of IL-10 in patients with ovarian carcinoma. Anticancer research. 2006;26(2C):1715-8.
24. Hirashima Y, Kobayashi H, Suzuki M, Tanaka Y, Kanayama N, Terao T. Transforming growth factor-beta1 produced by ovarian cancer cell line HRA stimulates attachment and invasion through an up-regulation of plasminogen activator inhibitor type-1 in human peritoneal mesothelial cells. J Biol Chem. 2003;278(29):26793-802.
25. Lechner MG, Megiel C, Russell SM, Bingham B, Arger N, Woo T, et al. Functional characterization of human Cd33+ and Cd11b+ myeloid-derived suppressor cell subsets induced from peripheral blood mononuclear cells co-cultured with a diverse set of human tumor cell lines. Journal of translational medicine. 2011;9:90.
26. Leontovich AA, Dronca RS, Suman VJ, Ashdown ML, Nevala WK, Thompson MA, et al. Fluctuation of systemic immunity in melanoma and implications for timing of therapy. Frontiers in bioscience. 2012;4:958-75.
27. French JD, Weber ZJ, Fretwell DL, Said S, Klopper JP, Haugen BR. Tumor-associated lymphocytes and increased FoxP3+ regulatory T cell frequency correlate with more aggressive papillary thyroid cancer. The Journal of clinical endocrinology and metabolism. 2010;95(5):2325-33.
28. Park HJ, Kusnadi A, Lee EJ, Kim WW, Cho BC, Lee IJ, et al. Tumor-infiltrating regulatory T cells delineated by upregulation of PD-1 and inhibitory receptors. Cell Immunol. 2012;278(1-2):76-83.
29. Schuler PJ, Schilling B, Harasymczuk M, Hoffmann TK, Johnson J, Lang S, et al. Phenotypic and functional characteristics of CD4+ CD39+ FOXP3+ and CD4+ CD39+ FOXP3neg T-cell subsets in cancer patients. Eur J Immunol. 2012;42(7):1876-85.
30. Chen ZF, Xu Q, Ding JB, Zhang Y, Du R, Ding Y. CD4+CD25+Foxp3+ Treg and TGF-beta play important roles in pathogenesis of Uygur cervical carcinoma. European journal of gynaecological oncology. 2012;33(5):502-7.

31. Chiba S, Baghdadi M, Akiba H, Yoshiyama H, Kinoshita I, Dosaka-Akita H, et al. Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. Nat Immunol. 2012;13(9):832-42.
32. Nunez S, Saez JJ, Fernandez D, Flores-Santibanez F, Alvarez K, Tejon G, et al. Th17 cells contribute to anti-tumor immunity and promote the recruitment of th1 cells to the tumor. Immunology. 2012.
33. Gao Y, Whitaker-Dowling P, Griffin JA, Bergman I. Treatment with targeted vesicular stomatitis virus generates therapeutic multifunctional anti-tumor memory CD4 T cells. Cancer gene therapy. 2012;19(4):282-91.
34. Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, et al. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. J Exp Med. 2010;207(3):637-50.
35. Ge Y, Domschke C, Stoiber N, Schott S, Heil J, Rom J, et al. Metronomic cyclophosphamide treatment in metastasized breast cancer patients: immunological effects and clinical outcome. Cancer immunology, immunotherapy : CII. 2012;61(3):353-62.
36. Jarosz M, Jazowiecka-Rakus J, Cichon T, Glowala-Kosinska M, Smolarczyk R, Smagur A, et al. Therapeutic antitumor potential of endoglin-based DNA vaccine combined with immunomodulatory agents. Gene therapy. 2012.
37. Rico MJ, Rozados VR, Mainetti LE, Fluck MF, Matar P, Scharovsky OG. Regulatory T cells but not NKT I cells are modulated by a single low-dose cyclophosphamide in a B cell lymphoma tumor-model. Experimental oncology. 2012;34(1):38-42.
38. van der Most RG, Currie AJ, Mahendran S, Prosser A, Darabi A, Robinson BW, et al. Tumor eradication after cyclophosphamide depends on concurrent depletion of regulatory T cells: a role for cycling TNFR2-expressing effector-suppressor T cells in limiting effective chemotherapy. Cancer immunology, immunotherapy : CII. 2009;58(8):1219-28.
39. de Vries IJ, Castelli C, Huygens C, Jacobs JF, Stockis J, Schuler-Thurner B, et al. Frequency of circulating Tregs with demethylated FOXP3 intron 1 in melanoma patients receiving tumor vaccines and potentially Treg-depleting agents. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011;17(4):841-8.

Part VI General discussion

Part V General discussion

General discussion

The current treatment of uterine tumors comprises surgery as the cornerstone of the first-line treatment, possibly followed by adjuvant treatments, such as chemotherapy, radiotherapy or hormonal therapy [1]. In case of far-advanced tumors or recurrent tumors, however, tumors often become treatment refractory and further treatment options are limited. Immunotherapy may provide an additional treatment option for these patients.

A major advantage of immunotherapy is that it is often accompanied by only minor non-toxic side-effects such as fatigue and only local reactions of the skin at the injection site. Recently used specific targeted treatments, such as ipilimumab, however, are accompanied with more severe side effects, which can be managed with the use of corticosteroids, administered simultaneously with ipilimumab in case of moderate side-effects [2]. In addition, a fairly large proportion of uterine tumor patients are elderly women, which advocates for the use of less toxic treatment options.

The **main goal** of the current project is the development of a dendritic cell-based vaccine for uterine tumors. Many different modalities can be applied for the composition of a dendritic cell vaccine, as described in the general introduction of this thesis, such as peptide-loaded DC, RNA-loaded DC or tumor lysate-loaded DC. We opted for the use of autologous monocyte-derived mRNA-electroporated DC. This technique offers several advantages. First of all, monocytes as an initial source for the generation of DC can easily be obtained via leukapheresis, followed by monocyte enrichment via plastic adherence or elutriation.

In addition, by using mRNA, patient selection for DC vaccination is no longer confined to patients with a certain HLA type. Antigens are processed by the DC's machinery into several peptides, each with its own MHC restriction. A certain peptide can only bind one specific type of MHC molecule, which results from the presence of the concomitant genetic HLA type. Thus, when using a certain peptide to load DC, only patients positive for the HLA type to which this peptide is restricted are eligible for vaccination. When using mRNA which encodes for the entire TAA, all possible peptides encoded by this antigen can be processed. Consequently, one is no longer bound to a patient's specific HLA subtype. In addition, mRNA that is used for electroporation can be artificially improved to obtain better results. The mRNA that was currently used was cloned into the pST1 vector, which was described to have better translational properties by Holtkamp [3]. In addition, the cDNA sequence was codon-optimized in order to obtain a more accurate transcription of the mRNA. Lastly, the cDNA sequence was coupled to a DC-LAMP sequence in order to ensure peptide presentation in both MHC I and MHC II context, ensuring the activation of helper T cells [4].

The development of this vaccine was subdivided in 2 objectives:

Obj. 1: Target antigen selection

An important lag in immunotherapy trials is the lack of a consistent correlation between an immunological response and a clinical response. An important reason that may explain this discrepancy is the presence of immunosuppressive mediators. Tumors often express immunosuppressive molecules on their surface, which can interact with immune cells in the tumor micro-environment and subsequently silence possible antitumor immune responses. In addition, uterine tumors, like all other tumor types, are often infiltrated with immune cells, further elaborated on in **chapter 2**, which can also contain immune cells with suppressive capacity, such as regulatory T

cells and myeloid-derived suppressor cells. This led us to the incorporation of the second objective in the project:

Obj. 2: Identification of immunosuppressive mechanisms

One other possible reason for a lack of immunological-clinical correlate is the evaluation criteria that are being used for the evaluation of the clinical response. This will be explained in further detail in the section with future directions.

OBJ. 1: TAA VALIDATION

The ideal tumor-associated antigen would be a highly specific, oncogenic antigen, with multiple epitopes ensuring the ability to bind most MHC molecules. It should be expressed by most tumor cells as well as cancer stem cells and by most tumors of a certain subtype. Additionally, the tumor antigen should be capable of eliciting a definite immune response and have proven therapeutic efficacy in clinical trials [5].

In the first part of this thesis, we validated the presence of arbitrarily chosen tumor-associated antigens (TAA). Based on the prioritization of cancer antigens, published in 2009 by Cheever et al. [5] as well as previously published data concerning TAA in uterine tumors and immunotherapeutic applications in other tumors, six different TAA were initially selected: BORIS, MUC1, survivin, MAGE-A3, Sp17 and hTERT (**chapter 3 and 4**). Sp17, BORIS and MAGE-A3 pose as interesting targets to investigate, due to their restricted expression in normal adult tissues. These molecules are members of the cancer testis antigen family and have an important function during development, but are restricted to expression in the male germ line in adult life. These antigens have been shown to re-emerge in cancer. Therefore, by targeting these antigens, one would be targeting the tumor specifically, while leaving normal surrounding tissues unharmed. The cancer testis antigens were found to be over-expressed in only a small fraction of the analyzed tumor samples, except for BORIS, which was expressed in 62% of uterine sarcomas.

Direct targeting of tumor survival is an important mode of action for anticancer therapies. Therefore, the antigens hTERT and survivin were selected additionally. These antigens are valid candidates for two main reasons. First, these antigens play a role in cell proliferation and survival during development. In normal, terminally differentiated adult cells and organs these antigens are virtually absent. In case of a growing tumor, these antigens are expressed in order to facilitate cell proliferation. Like for the cancer testis antigens, targeting these antigens is aimed at directly targeting the proliferating cancer cells, while not affecting normal non-proliferating cells. In addition, by directly targeting cell proliferation, tumor survival is affected. In the current analyses, we found expression of both these antigens in the majority of tumors. We also found high expression of MUC1 in EMCAR, and as expected in a substantially lower amount of US samples. MUC1 as a tumor antigen, however, cannot be implemented as an RNA construct. This is due to the fact that the TAA form of this antigen differs from the wild-type by a post-translational hypoglycosylation pattern, which cannot be incorporated in RNA encoding for MUC1.

Based on the expression profiles of these selected antigens, survivin and hTERT were further evaluated in DC. The quality of the DC that are used in the vaccine is of critical importance. The DC must be sufficiently capable of inducing a functional immune response. Electroporation with a

certain antigen may have an unfavorable effect on these capacities in terms of dendritic cell phenotype or function. Therefore, the effect of electroporation with survivin and hTERT was examined (unpublished results). Electroporation with hTERT proved unsuccessful, most likely due to the large size of the RNA construct for the molecule. Using 3 buffy coats from healthy donors (obtained from the Belgian Red Cross), we found that electroporation with survivin mRNA (obtained from the lab of molecular and cellular therapy, VUB , Brussels) did not result in a negative effect on DC phenotype, nor on its viability or functional capacity, as shown by their ability to elicit T cell proliferation in an allogeneic mixed leukocyte reaction (allo-MLR) (data not shown).

Based on the expression data and the effects on *in vitro* generated DC, in conjunction with the presence of spontaneous T cell responses in patient blood, it was decided to select survivin as a target antigen for further preclinical experiments for the development of the DC vaccine. Survivin was expressed in the vast majority of uterine tumor samples, and we detected spontaneous survivin-specific T cell responses in 22 % of endometrial cancer patients (4/18) and in 10 % (1/10) of uterine sarcoma patients.

OBJ. 2: IMMUNOSUPRESSIVE ENVIRONMENT

An important issue hampering the efficacy of immunotherapy is that of immunosuppression. This can be the result of either the intratumoral immune infiltrate, which may contain suppressive cells such as MDSC and macrophages, or Treg, or the presence of immune mediators on tumor cells or in the tumor microenvironment. On the contrary to ovarian cancer, little information is currently available concerning the immunosuppressive environment in endometrial cancer (**Part I, chapter 2**).

We have explored the presence of several important immunosuppressive mediators (**Part III, chapter 1**) in uterine tumors. We found expression of several members of the B7 family. We have currently analyzed the presence of PD-L1 (B7-H1), PD-L2 (B7-DC), and B7-H4, as well as IDO, galectin-1 and galectin-3 and the presence of intratumoral MDSC and MDSC activity.
We identified the PD-1/PD-L1 axis as well as B7-H4 as possible targets for therapeutic intervention, since PD-L1 and B7-H4 were expressed in vast majority of the current tumor sample population. In addition, in a small portion of both endometrial cancer and uterine sarcoma, IDO presented as a valid option.

The current data are, to our knowledge, the first data describing the presence of MDSC in uterine tumors. We found both monocytic and granulocytic MDSC infiltration in uterine tumors. In addition, Arginase-1 activity was measured at increased levels in endometrial tumors compared to normal endometrial tissues, indicating that the MDSC are most likely functionally active. Thus, mediation of MDSC function can be exploited in the treatment of endometrial cancer.

CLINICAL APPLICATION AND SITUATION IN CURRENT LANDSCAPE OF IMMUNOTHERAPY FOR UTERINE TUMORS

Although having been applied in many different cancer types, immunotherapy in uterine tumors has not been explored to much extent so far. To our knowledge, less than 15 clinical studies have been published to date (Table 1). As shown in Table 1, current studies in uterine tumors result in no or transient clinical responses, indicating that there are still many hurdles to overcome to increase the efficacy of immunotherapy in uterine tumors, as is the case in other tumor types.

Most studies have been done using peptides (6/13). Only 3 studies and 1 case report using DC therapy were found. To our knowledge, the current setup of mRNA-based DC therapy is so far only pursued in uterine tumors by our own research group.

The current results are in any case a step forward in the research by our own research group [5, 6]. Our results have identified survivin as a novel antigenic target to include in the vaccine, beside the previously validated WT1. This will further strengthen the DC vaccine, since now 2 separate antigens are targeted instead of a single antigen, and it is aimed at directly targeting the survival of the tumor, since survivin is actively involved in tumor cell proliferation and survival. In addition, the RNA constructs that were validated preclinically are transcribed from codon-optimized cDNA, ensuring transcription of the optimal cDNA sequence of the target antigen and thus provides an optimal starting point for successful protein translation of the desired antigen. In addition, the mRNA was coupled to the DC-LAMP signal, which ensures antigen presentation in both MHC I and MHC II context [4]. This set-up was already applied for 1 patient in the first cohort in our own study [5]. In addition, the current results using TriMix for DC maturation have shown that this maturation cocktail results in DC of good phenotypic quality. TriMix DC have been used previously in clinical trials in melanoma patients [7-9]. In 71.4 % of tested patients, TAA-reactive T cells could be found both in blood and in a skin biopsy taken at the site of vaccine injection [7]. Another study showed that using a mixture of TriMix DC loaded with four different antigens, in combination with IFN-α-2b, durable clinical responses could be measured in 35 % of patients. In 1/17 patients, partial disease remission was seen and in 5 other patients, stable disease lasting more than 6 months was seen [9].

Table 1: Immunotherapy trials in uterine tumors (adapted from [10]).

Author	Tumor type	Trial phase	Set-up	Number of patients	Outcome
Steis et al. [11]	Endometrial carcinoma	Phase I	ACT of activated killer cells + interperitoneal IL-2	1	PD
Hersh et al. [12]	Endometrial carcinoma	Phase II	Injection with TNF-α	2	Not specified
Santin et al. [13]	Endometrial carcinoma	Case report	T cells stimulated *in vitro* with tumor lysate-loaded DC		Tumor marker stabilization, intratumoral T cell infiltration
Santin et al. [14]	Endometrial carcinoma	Phase I	DC loaded with whole tumor lysate	3	Induction of $CD8^+$ T cells with *in vitro* killing capacity
Tsuda et al. [15]	Endometrial carcinoma	Phase I	Injection of up to 4 different peptides	1	PD 2 months after start of therapy
Kaumaya et al. [16]	Endometrial carcinoma	Phase I	Injection of B cell epitopes fused to a promiscuous T cell epitope	2	½ patients in partial remission for 4 years
Ohno et al. [17]	Endometrial cancer	Phase I	Injection with a modified WT1 peptide	1	PD 3 months after start of therapy
Coosemans et al. [6]	Endometrial carcinoma	Case report	WT1 mRNA-loaded DC		Tetramer-positive T cells post vaccination
Coosemans et al. [5]	Endometrial carcinoma/uterine sarcoma	Phase I/II	WT1 mRNA-loaded DC	6	Immunological response in 3/6 patients. Transient clinical responses in 2/6 patients
Miyatake et al. [18]	Uterine sarcoma	Phase II	WT1 peptide vaccination	5	Not specified
Hernando et al. [19]	Uterine sarcoma	Phase I	DC loaded with tumor lysate and KLH	2	PD after 3 months in 1 patient and 6 months in patient 2
Tsuda et al. [15]	Uterine sarcoma	Phase I	Injection of up to 4 peptides	1	PD after 5 months
Ohno et al. [17]	Uterine sarcoma	Phase I	WT1 peptide	1	PD after 3 months

The current data will be implemented in a clinical trial, using TriMix DC. Initial proof of concept data have shown that 48h after electroporation, DC obtain a mature phenotype, with expression of CD54, CD80, CD83, CD86, and CCR7 [20].

As research progresses, gradually more and more mechanisms of immunosuppression are being unraveled, raising awareness of the importance of these mechanisms. This results in more and more efforts being done to combine active immunotherapy and targeting of immunosuppression. The

current results provide important information on which targets to be used in future combination therapies in uterine tumors.
For these targets, several inhibitors are currently being tested in clinical settings, as elaborated on in the discussion to **Part III, chapter 1** and **Part I, chapter 1**.

FUTURE DIRECTIONS

The current work is the expansion of previous research regarding WT1 as an immunotherapeutic target in uterine cancer [6, 21-23]. We have currently selected an additional antigen to include in the vaccine. The currently validated targets were arbitrarily chosen, mainly based on literature. However, these are not the only valid targets. Future expansion of the current findings may be accomplished by further validation of additional targets or screening for new tumor-associated antigens.

In addition to using a (combination of) single, defined antigens, total tumor RNA presents as another option for antigen loading. With this technique, all tumor-associated antigens are included, including the tumor driver genes. In addition, tumor-specific epitopes that differ slightly from the natural counterparts, due to genetic mutations in a specific tumor can also be presented to the immune system (mutatopes). The latter provides the benefit that no tolerance exists against mutatopes, since many conventional TAA are in fact auto-antigens and thus subject to tolerogenic mechanisms.

One factor contributing to the occurrence of recurrent tumors, is that of cancer stem cells. Vaccination with moDC loaded with total tumor RNA from glioma cancer stem cells was recently shown to result in progression-free survival that was 2.9 times as high as that of normal controls [24]. Evidently, when using total tumor mRNA, mRNA originating from cancer stem cells is included, which may consequently contribute to targeting of cancer stem cells.
A downside of using whole tumor mRNA is that electroporation will result in antigen presentation in MHC I context only. In addition, whole tumor mRNA presents with some difficulties regarding immune monitoring. In case of whole tumor mRNA antigen-specific immune monitoring is not feasible since the target antigen that can be used for monitoring cannot be readily identified. This can be overcome by adding mRNA encoding for a defined TAA coupled to the DC-LAMP signal. This will ensure both presentation in MHC I and MHC II context of the defined TAA and thus provide $CD4^+$ T cell help.
Some studies have been performed using total tumor mRNA. A preclinical study using total mRNA from a mouse gastric cancer cell line to load dendritic cells together with 4-1BBL, has shown that it induces a stronger T cell proliferation than DC alone, as well as an increased secretion of IL-12 and increased killing capacity towards MFC target cells [25]. In addition, autologous DC transfected with total tumor RNA could induce antigen-specific T cell responses *in vivo* in patients [26], indicating that this approach holds promise for future clinical applications.

In current clinical trials, there is often an important lack of a clinical correlate with a detected immune response. The immunosuppressive environment is an important factor influencing this parameter, although the reported lack may also be explained in part at a different level. The immunological outcome is highly dependent on the immunological parameters that are being assessed. The immune system has a complex role in cancer, including several players, which can have both anti-tumor and pro-tumor functions. Using the proper targets for monitoring can improve the effectiveness of the monitoring and may clarify the correlation between a clinical and immunological

response. In addition, next to the parameters that are included in the immunological monitoring, the site at which the monitoring is performed is of importance. Many immune monitoring assays are being performed in peripheral blood. However, this may generate a certain bias since the site of action for the immune system is the tumor site. By comparing intratumoral factors or immune factors in the tumor microenvironment prior to and after immunotherapy, a more adequate analysis can be obtained [27].

Clinical responses are currently evaluated using the commonly applied and validated RECIST criteria. However, these criteria may potentially not suffice for the evaluation of a clinical anti-tumor response. Immunotherapy can results in an initial increase in tumoral lesions, followed by a subsequent decline after a certain amount of time. Consequently, an increase in existing lesions or the occurrence of new lesions is considered to be a part of the tumor burden and does not necessarily indicate progressive disease (PD) according to immune-related criteria [28]. A comparison between the WHO, RECIST and immune-related response criteria (irRC) is given in Table 2 [29].

Table 2: Comparison between WHO, RECIST and irRC (adapted from [29].

	WHO	**RECIST**	**irRC**
CR	No lesion detected for at least 4 weeks	Disappearance of all target lesions or lymph nodes <10 mm in the short axis	Disappearance of all lesions (including non-index lesions) at two consecutive observations ≥ 4 weeks apart, no new nonmeasurable disease
PR	≥ 50% decrease in SPD (confirmed at 4 weeks)	> 30% decrease in SLD of target lesions	50 % decrease in tumor burden (confirmed at 4 weeks)
PD	≥ 25% increase in SPD in one or more lesions; new lesions	> 20% increase in SLD of target lesions with an absolute increase of 5 mm; new lesions	25% increase in tumor burden (confirmed at 4 weeks), new measurable lesions included within tumor burden
SD	None of the above	None of the above	None of the above

SPD: sum of perpendicular diameters. SLD: sum of longest diameters.

As stated above, more efforts are being put in the development of combination therapies to target cancer, often involving the immune system. To this end, many options can be pursued.
A preclinical study using a combination therapy of a DC vaccine loaded with tumor lysate originating from breast cancer cell lines and anti PD-L1 antibody in mice showed that the combination of the DC vaccine and anti PD-L1 slowed tumor growth and survival of the mice compared to the untreated group. Interestingly, the results were even more profound when anti PD-L1 was used during the maturation of DC as well as in combination with a vaccine using these DC [30]. Currently (May 2014), 353 clinical trials evaluating combination immunotherapy in cancer are listed at clinicaltrials.gov. These trials include trials combining active immunotherapy and targeted therapy, immunotherapy and chemotherapy, whether or not in combination with immune checkpoint inhibitors, and combinations of immunogenic chemotherapy.

Beside targeted treatments or known cytotoxic anticancer therapies, some food supplements may also have anticancer effects and can also be used in immunotherapeutic strategies. Several groups of nutrient components have been shown to have antitumor effects, reviewed by González-Vallinas et al. [31]. The most familiar of these nutrients are flavonoids, mainly found in fruit and green tea, β-carotene present in carrots and curcuminoids, found in the spice turmeric [31].
These supplements together with for example nutrients to fortify the patients' immune system may also be incorporated in future immunotherapy-related trials.

Collectively, the current research builds on and further extends previous research by our own research group on WT1 as an immunotherapeutic target and provides the basis for a subsequent clinical study. However, immunotherapy in uterine tumors is still in its early days and much is still left to be explored. With the current data, we have increased the, as of yet still quite narrow, insights in the immune system in uterine tumors, providing another up-step for future exploratory and treatment-directed research in uterine cancer.

REFERENCES

1. Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E, Vergote I. Endometrial cancer. Lancet. 2005;366(9484):491-505.
2. Andrews S, Holden R. Characteristics and management of immunerelated adverse effects associated with ipilimumab, a new immunotherapy for metastatic melanoma. Cancer management and research. 2012;4:299-307.
3. Holtkamp S, Kreiter S, Selmi A, Simon P, Koslowski M, Huber C, et al. Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells. Blood. 2006;108(13):4009-17.
4. Bonehill A, Heirman C, Tuyaerts S, Michiels A, Breckpot K, Brasseur F, et al. Messenger RNA-electroporated dendritic cells presenting MAGE-A3 simultaneously in HLA class I and class II molecules. Journal of immunology. 2004;172(11):6649-57.
5. Coosemans A, Vanderstraeten A, Tuyaerts S, Verschuere T, Moerman P, Berneman ZN, et al. Wilms' Tumor Gene 1 (WT1)--loaded dendritic cell immunotherapy in patients with uterine tumors: a phase I/II clinical trial. Anticancer research. 2013;33(12):5495-500.
6. Coosemans A, Wolfl M, Berneman ZN, Van Tendeloo V, Vergote I, Amant F, et al. Immunological response after therapeutic vaccination with WT1 mRNA-loaded dendritic cells in end-stage endometrial carcinoma. Anticancer research. 2010;30(9):3709-14.
7. Benteyn D, Van Nuffel AM, Wilgenhof S, Corthals J, Heirman C, Neyns B, et al. Characterization of CD8+ T-cell responses in the peripheral blood and skin injection sites of melanoma patients treated with mRNA electroporated autologous dendritic cells (TriMixDC-MEL). BioMed research international. 2013;2013:976383.
8. Van Nuffel AM, Benteyn D, Wilgenhof S, Corthals J, Heirman C, Neyns B, et al. Intravenous and intradermal TriMix-dendritic cell therapy results in a broad T-cell response and durable tumor response in a chemorefractory stage IV-M1c melanoma patient. Cancer immunology, immunotherapy : CII. 2012;61(7):1033-43.
9. Wilgenhof S, Van Nuffel AM, Corthals J, Heirman C, Tuyaerts S, Benteyn D, et al. Therapeutic vaccination with an autologous mRNA electroporated dendritic cell vaccine in patients with advanced melanoma. Journal of immunotherapy. 2011;34(5):448-56.
10. Coosemans A, Tuyaerts S, Vanderstraeten A, Amant F. Immunotherapy in uterine cancers. In: Ayhan A, Reed N, Gultekin M, Dursun P, editors. textbook of gynaecological oncology. 2nd ed. Ankara: Günes Publishing; 2010. p. 809-16.
11. Steis RG, Urba WJ, VanderMolen LA, Bookman MA, Smith JW, 2nd, Clark JW, et al. Intraperitoneal lymphokine-activated killer-cell and interleukin-2 therapy for malignancies limited to

the peritoneal cavity. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1990;8(10):1618-29.
12. Hersh EM, Metch BS, Muggia FM, Brown TD, Whitehead RP, Budd GT, et al. Phase II studies of recombinant human tumor necrosis factor alpha in patients with malignant disease: a summary of the Southwest Oncology Group experience. Journal of immunotherapy : official journal of the Society for Biological Therapy. 1991;10(6):426-31.
13. Santin AD, Hermonat PL, Ravaggi A, Bellone S, Cowan C, Coke C, et al. Development and therapeutic effect of adoptively transferred T cells primed by tumor lysate-pulsed autologous dendritic cells in a patient with metastatic endometrial cancer. Gynecologic and obstetric investigation. 2000;49(3):194-203.
14. Santin AD, Bellone S, Ravaggi A, Roman JJ, Pecorelli S, Parham GP, et al. Induction of tumour-specific CD8(+) cytotoxic T lymphocytes by tumour lysate-pulsed autologous dendritic cells in patients with uterine serous papillary cancer. British journal of cancer. 2002;86(1):151-7.
15. Tsuda N, Mochizuki K, Harada M, Sukehiro A, Kawano K, Yamada A, et al. Vaccination with predesignated or evidence-based peptides for patients with recurrent gynecologic cancers. Journal of immunotherapy. 2004;27(1):60-72.
16. Kaumaya PT, Foy KC, Garrett J, Rawale SV, Vicari D, Thurmond JM, et al. Phase I active immunotherapy with combination of two chimeric, human epidermal growth factor receptor 2, B-cell epitopes fused to a promiscuous T-cell epitope in patients with metastatic and/or recurrent solid tumors. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2009;27(31):5270-7.
17. Ohno S, Kyo S, Myojo S, Dohi S, Ishizaki J, Miyamoto K, et al. Wilms' tumor 1 (WT1) peptide immunotherapy for gynecological malignancy. Anticancer research. 2009;29(11):4779-84.
18. Miyatake T, Ueda Y, Morimoto A, Enomoto T, Nishida S, Shirakata T, et al. WT1 peptide immunotherapy for gynecologic malignancies resistant to conventional therapies: a phase II trial. Journal of cancer research and clinical oncology. 2013;139(3):457-63.
19. Hernando JJ, Park TW, Kubler K, Offergeld R, Schlebusch H, Bauknecht T. Vaccination with autologous tumour antigen-pulsed dendritic cells in advanced gynaecological malignancies: clinical and immunological evaluation of a phase I trial. Cancer immunology, immunotherapy : CII. 2002;51(1):45-52.
20. Coosemans A, Tuyaerts S, Vanderstraeten A, Vergote I, Amant F, Van Gool SW. Dendritic cell immunotherapy in uterine cancer. Human vaccines & immunotherapeutics. 2014;10(7).
21. Coosemans A, Moerman P, Verbist G, Maes W, Neven P, Vergote I, et al. Wilms' tumor gene 1 (WT1) in endometrial carcinoma. Gynecologic oncology. 2008;111(3):502-8.
22. Coosemans A, Nik SA, Caluwaerts S, Lambin S, Verbist G, Van Bree R, et al. Upregulation of Wilms' tumour gene 1 (WT1) in uterine sarcomas. European journal of cancer. 2007;43(10):1630-7.
23. Coosemans A, Van Calster B, Verbist G, Moerman P, Vergote I, Van Gool SW, et al. Wilms tumor gene 1 (WT1) is a prognostic marker in high-grade uterine sarcoma. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society. 2011;21(2):302-8.
24. Vik-Mo EO, Nyakas M, Mikkelsen BV, Moe MC, Due-Tonnesen P, Suso EM, et al. Therapeutic vaccination against autologous cancer stem cells with mRNA-transfected dendritic cells in patients with glioblastoma. Cancer immunology, immunotherapy : CII. 2013;62(9):1499-509.
25. Song Z, Guo C, Li Y, Tan B, Fan L, Xiao J. Enhanced antitumor effects of a dendritic cell vaccine transfected with gastric cancer cell total RNA carrying the 4-1BBL gene in vitro. Experimental and therapeutic medicine. 2012;3(2):319-23.
26. Nair SK, Morse M, Boczkowski D, Cumming RI, Vasovic L, Gilboa E, et al. Induction of tumor-specific cytotoxic T lymphocytes in cancer patients by autologous tumor RNA-transfected dendritic cells. Annals of surgery. 2002;235(4):540-9.
27. Malyguine AM, Strobl SL, Shurin MR. Immunological monitoring of the tumor immunoenvironment for clinical trials. Cancer immunology, immunotherapy : CII. 2012;61(2):239-47.

28. Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbe C, et al. Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15(23):7412-20.
29. Tirkes T, Hollar MA, Tann M, Kohli MD, Akisik F, Sandrasegaran K. Response criteria in oncologic imaging: review of traditional and new criteria. Radiographics : a review publication of the Radiological Society of North America, Inc. 2013;33(5):1323-41.
30. Ge Y, Xi H, Ju S, Zhang X. Blockade of PD-1/PD-L1 immune checkpoint during DC vaccination induces potent protective immunity against breast cancer in hu-SCID mice. Cancer letters. 2013;336(2):253-9.
31. Gonzalez-Vallinas M, Gonzalez-Castejon M, Rodriguez-Casado A, Ramirez de Molina A. Dietary phytochemicals in cancer prevention and therapy: a complementary approach with promising perspectives. Nutrition reviews. 2013;71(9):585-99.

Part VII Summary

Summary

Although current treatments are often curative for uterine cancer when it is diagnosed in an early stage, with a 5-year survival rate of around 80 %, these treatments are often no longer effective in case of far-advanced disease. The mean survival rate for stage III uterine tumors is 57.8 %, while for stage IV, this number decreases even further to a mere 22.8 %. Consequently, additional treatments are needed.

The main focus of the current project was to further develop an autologous dendritic cell-based vaccine for uterine tumors. The initial vaccine, using WT1 mRNA loaded monocyte-derived DC, showed an immunological response in 5/8 patients and a transient clinical response in 2/8 patients (**chapter 6)**. In the current study, the vaccine was further improved and extended.

The first objective was to identify additional antigenic targets to include in the vaccine. We selected 6 different antigens, MAGE-A3, BORIS, MUC1, Sp17, hTERT and survivin, for validation using immunohistochemistry as well as qRT-PCR. In addition, to verify the immunogenicity of these antigens, spontaneous *ex vivo* T cell responses to these antigens were analyzed. From these analyses, we found that survivin was the best suitable target to proceed with (**Chapter 3 and 4**).
In addition to adding a new target antigen to the DC vaccine, we aimed to further improve the functional characteristics of the DC used in the vaccine. To this end, an optimal protocol for the generation of monocyte-derived DC was established using an IL-4/GM-CSF/TriMix maturation cocktail. As shown in previously published data this improves the maturity profile of the DC and improves the T cell stimulatory capacity.
Tumor-induced immunosuppression is an important factor hampering the efficacy of immunotherapy. More and more awareness is being raised for this issue. We aimed to map some of the immunosuppressive mechanisms in uterine tumors. To this end, 3 members of the B7 family, B7-H1 (PD-L1), B7-DC (PD-L2) and B7-H4 were analyzed as well as IDO, galectin-1 and galectin-3 as well as the presence and activity of myeloid-derived suppressor cells in uterine tumors (**chapter 5**). IHC analysis showed that the PD-1:PD-L1 axis and B7-H4 are promising targets in the vast majority of tumors and IDO in a minority of cases. Flow cytometric analysis showed that MDSC do infiltrate uterine tumors and are likely functionally active, thus presenting another route at which tumor immune escape may be interrupted.

Beside using active immunotherapeutic regimens or direct intervention of immunosuppression, substances that are used for current anti-cancer treatment may also be averted in a different dosage to modulate the immune system. Low-dose cyclophosphamide, for example, has been shown to have immunomodulatory effects. We verified the effect of oral administration of low-dose cyclophosphamide on the Treg population in order to determine whether or not this modality could be applied for uterine tumors. However, we could not confirm the suppressive effect of cyclophosphamide on Treg (**chapter 7**). Consequently, this method is, according to our results, not applicable in uterine tumors.
Taken together, the current research is the next step in the identification of the role the immune system plays in uterine tumors and provides novel indications for its use and its modulation in the treatment of uterine cancer.

Samenvatting

De huidige behandeling voor uteriene tumoren is in de meerderheid van de gevallen een curatieve behandeling wanneer de tumor gediagnosticeerd is in een vroeg stadium. De overlevingskans na 5 jaar bedraagt hier 80 %. In een vergevorderd stadium daarentegen, zijn de huidige behandelingen echter niet meer effectief. De 5 jaar overleving bedraagt 57.8 % voor stadium III tumoren en slechts 22.8 % voor stadium IV tumoren. Bijgevolg is er een hoge nood aan bijkomende behandeling.

De voornaamste focus van het huidige project is de ontwikkeling van een autoloog dendritische cel-gebaseerd vaccin voor uteriene tumoren. Het initiële vaccin ontwikkeld door onze onderzoeksgroep, waarbij er gebruik gemaakt werd van DC afgeleid van monocyten (moDC), opgeladen met mRNA coderend voor WT1, resulteerde na behandeling in een immunologische respons in 5/8 patiënten en een transiënte klinische respons in 2/8 patiënten (**hoofdstuk 6**). In de huidige studie werd dit vaccin verder uitgebreid en verbeterd.

Het eerste objectief van de studie behandelde de identificatie en de validatie van bijkomende antigen doelwitten om te includeren in het vaccin. Zes antigenen werden geselecteerd, MAGE-A3, BORIS, MUC1, Sp17, hTERT en survivine, voor validatie door middel van IHC en qRT-PCR. Bijkomend werden de *ex vivo* spontane T cel responsen gericht tegen deze antigenen nagegaan. Uit deze resultaten werd survivine geïdentificeerd als bijkomend doelwitantigen voor het vaccin (**hoofdstuk 3 en 4**).
Bijkomend aan het toevoegen van een doelwitantigen, beoogden we tevens de functionele karakteristieken van het bestaande vaccin verder te verbeteren. Hiervoor werd een optimaal protocol ontwikkeld voor de generatie van moDC, volledig gematureerd met een IL-4/GM-CSF/TriMix cocktail. Eerder gepubliceerde data toonden aan dat met deze cocktail een verbeterd maturiteitsprofiel en een verhoogde T cel stimulatie kan bereikt worden.
Tumor-geïnduceerde immuun suppressie is een belangrijke factor in de belemmering van immunotherapeutische behandelingen. Dit probleem wordt steeds meer onder de aandacht gebracht. Een volgend doel in het project was om een deel van deze immuunmodulerende factoren in uteriene tumoren in kaart te brengen. Drie componenten van de B7 familie, B7-H1 (PD-L1), B7-DC (PD-L2) en B7-H4 werden geanalyseerd, naast IDO, galectine-1 en galectine-3. De aanwezigheid en functionele activiteit van MDSC in uteriene tumoren werd eveneens bepaald (**hoofdstuk 5**). IHC analyse toonde aan dat de PD-1:PD-L1 as en B7-H4 veelbelovende doelwitten zijn in de meerderheid van de tumoren en IDO in een minderheid van de gevallen. Een andere mogelijke route voor therapeutische interventie is deze van de MDSC, aangezien deze functioneel actief aangetoond worden in uteriene tumoren.

Naast actieve immunotherapeutische strategieën of directe interventie van immuun suppressieve mechanismen, kunnen therapeutica die momenteel gebruikt worden in de standaard kankerbehandeling in een aangepaste dosis aangewend worden om het immuunsysteem te moduleren. Voor cyclofosfamide is er reeds eerder aangetoond dat er in lage dosis gunstige effecten op het immuunsysteem zijn, met name de depletie en functionele onderdrukking van Treg. Deze effecten konden echter niet bevestigd worden in patiënten met gynaecologische tumoren (**hoofdstuk 7)**.

Het huidige onderzoek vormt de volgende stap in het ontrafelen van de rol die het immuun systeem speelt in uteriene tumoren en toont nieuwe indicaties aan voor het aanwenden en het moduleren van het immuun systeem in de behandeling van uteriene tumoren.

Professional career

Birth: Hasselt, September 25th 1986

High school education: Latin-mathematics.
1998 – 2004. Sint-Aloysius Instituut, Zepperen

Higher education: Biomedical sciences, subspeciality clinical molecular lifesciences
Hasselt University. 2004 – 2009

Current degree: Master of Science in biomedical sciences

SCIENTIFIC ABSTRACTS

- **Vanderstraeten A.** et al. $CD4^{+}CD28^{NULL}$ T cells in autoimmune disease. Culprits or bystanders?
  Maastricht Medical Students Research Conference. 2009, Maastricht, the Netherlands.

- **Vanderstraeten A.** et al. The role of $CD4^{+}CD28^{NULL}$ T cells in the pathogenesis of multiple sclerosis and rheumatoid arthritis.
  International Life Sciences Master students Research Conference, 2009. Maastricht, the Netherlands.

- **Anke Vanderstraeten,** Sandra Tuyaerts, Catherine Luyten, Rieta Van Bree, Lieve Verbist, Carlo Heirman, Kris Thielemans, Frédéric Amant. Tumor-associated antigen validation in uterine tumors.
  Oncoforum, 2012. Leuven, Belgium.

- Sandra Tuyaerts, **Anke Vanderstraeten**, Catherina Luyten, Rieta Van Bree, Godelieve Verbist, Frederic Amant. Validation of tumour antigens for immunotherapy of uterine cancer.
  Cancer Research Institute conference, 2012. New York, U.SA.

- Sandra Tuyaerts, An Coosemans, **Anke Vanderstraeten**, Zwi N Berneman, Vigor FI Van Tendeloo, Ignace Vergote, Stefaan W Van Gool, Frederic Amant. WT1-specific dendritic cell vaccination in uterine cancer
  Cancer Research Institute conference, 2012, New York, U.SA.

- **Anke Vanderstraeten,** Sandra Tuyaerts, Catherine Luyten, Rieta Van Bree, Lieve Verbist, Carlo Heirman, Kris Thielemans, Frederic Amant. Tumor-associated antigen validation in uterine tumors.
  European Association for Cancer Research conference, 2012. Barcelona, Spain.

- Sandra Tuyaerts, An MT Van Nuffel, **Anke Vanderstraeten**, Tina Everaert, Carlo Heirman, Kris Thielemans and Frederic Amant Effect of rapamycin and curcumin on TriMix DC.
  Association for cancer immunotherapy conference, 2013. Mainz, Germany.
  Oncoforum, 2013. Leuven, Belgium.

- Sandra Tuyaerts, **Anke Vanderstraeten**, Godelieve Verbist, Rieta Van Bree,Tina Everaert and Frederic Amant. Tumor-infiltrating immune cell profiling in uterine tumors –implications for immune therapy.
  Cancer Immunology and Immunotherapy conference, 2013. Krakow, Poland.
  Oncoforum, 2013. Leuven, Belgium.

- Sandra Tuyaerts, **Anke Vanderstraeten**, Tina Everaert, Godelieve Verbist, Rieta Van Bree, Frederic Amant. Phenotypic characterization of the immune infiltrate in gynecological tumors.
  Cancer research Institute conference, 2013. New York, USA.

- Sandra Tuyaerts, **Anke Vanderstraeten**, Tina Everaert, Frederic Amant. Phenotypic characterization of the immune infiltrate in gynaecological tumours.
  Association for cancer immunotherapy conference, 2014. Mainz, Germany.
  Oncoforum, 2014. Leuven, Belgium.

- Sandra Tuyaerts, An Coosemans, Kim Morias, Tina Everaert, **Anke Vanderstraeten**, Stefaan W. Van Gool, Frederic Amant. Exploring dendritic cell (DC) vaccines targeting Wilms' tumour gene 1 (WT1) and survivin for uterine cancer.
  Association for cancer immunotherapy conference, 2014. Mainz, Germany.
  Oncoforum, 2014. Leuven, Belgium.

Publications

PUBLICATIONS

Non-peer reviewed book chapter

Coosemans, A., S. Tuyaerts, **A. Vanderstraeten**, and F. Amant. 2012. Immunotherapy in uterine cancers. In Textbook of gynecological oncology, 2 ed. A. Ayhan, N. Reed, M. Gultekin, and P. Dursun, eds. Günes Publishing, Ankara, Turkey. 809-816.

Peer-reviewed journal papers, related to PhD

Coosemans A, **Vanderstraeten A**, Tuyaerts S, Verschuere T, Moerman P, Berneman Z, et al. Immunological response after WT1 mRNA-loaded dendritic cell immunotherapy in ovarian carcinoma and carcinosarcoma. Anticancer res. 2013;33(9):3855-9.

Coosemans A, **Vanderstraeten A**, Tuyaerts S, Verschuere T, Moerman P, Berneman ZN, et al. Wilms' Tumor Gene 1 (WT1)-loaded dendritic cell immunotherapy in patients with uterine tumors: a phase I/II clinical trial. Anticancer res. 2013;33(12):5495-500.

Vanderstraeten A, Luyten C, Verbist G, Tuyaerts S, Amant F. Mapping the immunosuppressive environment in uterine tumors: implications for immunotherapy. Cancer Immunol Immunother. 2014;63(6):545-57.

Madondo MT, Tuyaerts S, Turnbull BB, **Vanderstraeten A**, Kohrt H, Narasimhan B, Amant F, Quinn M, Plebanski M. Variability in CRP, regulatory T cells and effector T cells over time in gynaecological cancer patients: a study of potential oscillatory behaviour and correlations. J Transl Med. 2014; 23;12(1):179.

Peer-reviewed journal papers, not related to PhD

Coosemans A, Tuyaerts S, **Vanderstraeten A**, Vergote I, Amant F, Van Gool SW. Dendritic cell immunotherapy in uterine cancer. Hum vaccin immunother. 2014;10(7).

SUBMITTED MANUSCRIPTS/MANUSCRIPTS IN PREPARATION

Vanderstraeten A, Tuyaerts S and Amant F. The immune system in the normal uterus and its implication in endometrial cancer development. *Submitted to J Reprod Immunol.*

Vanderstraeten A, Tuyaerts S, Everaert T, Van Bree R, Verbist G, Luyten C and Amant F. Validation of tumor antigens as immunotherapeutic targets in uterine cancer. *Manuscript in preparation.*

Vanderstraeten A, Tuyaerts S, Everaert T, Van Bree R, Verbist G, Luyten C and Amant F. Preclinical evaluation of survivin as target tumor-associated antigen for immunotherapy in uterine cancer. *Submitted to J Immunoth.*